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DETERMINATION OF GENE EXPRESSION OF CD25 AND CD29 IN ALBINO RATS IMMUNIZED WITH VI ANTIGEN OF *SALMONELLA TYPHI* COUPLED TO CHITOSAN NANOPARTICLES AND TETANUS TOXOID.

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ABSTRACT: *Typhoid fever is an important enteric pathogen causing Salmonella typhi, is still extremely common in developing countries of the world. Therefore, this study was an attempt to develop an experimental vaccine against typhoid fever through determination of the immunogenic properties of Vi antigen when loaded on chitosan and tetanus toxoid in laboratory animals. Recorded results showed that the antibody titers were significantly elevated: in (T1) Vi Ag + chitosan groups(620.8±364.8) increased significantly than other groups; (T2) Vi Ag group (448±117.73), (T3) Vi + Tetanus toxoid group (537.6±142.53) and (T4) Vi Ag+ Ch. +TT group (121.6±38.4) at (P≤ 0.05). The gene expression of CD25 and CD29 was done by RT-qPCR . The results of relative gene expression in CD25 gene showed that in T4 group (5.1936±2.17) was increased significantly than other groups, which were; T1 (2.5604±0.64), T2 (3.7032±0.54) and T3 (4.4739±1) at (P≤ 0.05). The results of relative gene expression in CD29 gene showed that T3group recorded significantly increment (162.3256±89.52) than other groups, which were; T1 (33.8276±16.6), T2 (59.6817±26.65), and T4 (37.9605±3.09) at (P≤ 0.05). This findings were supported by the increment of total white blood cells in immunized animals and was clearly determined in lymph nodes of spleen & thymus which increased in size and numbers which active germinal centers.*

KEYWORDS: Chitosan nanoparticles, Vi antigen, Tetanus Toxoid, CD25 marker,CD29 marker .

INTRODUCTION

Salmonella enterica serovar typhi (*S. typhi*), the etiologic agent of typhoid fever, is a human restricted pathogen (Kaur&Jain, 2012). Typhoid fever is one of the most important food borne disease , is a severe systemic disease with an estimated incidence of ~22 million cases per year (Maurice, 2012). Vaccination against such disease remain the most effective and cost-efficient means to prevent infectious, Vaccines against typhoid are commonly used by travelers but less so by residents of endemic areas .The latest trend towards novel and safer vaccines utilize well characterized antigens ,which can enable the immune system to respond to the desired specificity without the risks , Unfortunately, such subunit antigens are often poor immunogens when administered (Holt *et al*.,2012).Therefore, an adjuvant is required to potentiate the immune response to the coadministrated antigen .Vi antigen of *S.typhi* is a capsular polysaccharide found mainly in *Salmonella typhi* and *S.paratyphiC* ,as well as in a few strains of *S.dublin* and *Citrobacter freundii* ,this antigen has been shown to be protective against

typhoid fever (Kothari *et al*., 2013; Felix &Pitt ,1936).Vi antigen is made of repeating units of (1–4)-2-deoxy-2-N-acetyl galacturonic acid,60–90% O-acetylated at the C-3 position and is encoded within the viaB locus from *S.typhi* (Raffatellu *et al*.,2008). Chitosan presents biological characteristics such as low or no toxicity, biocompatibility, biodegradability, low immunogenicity and antimicrobial properties as well as linear nitrogenous polysaccharides, a basic polysaccharide homo-polymer (Hayes *et al*.,2008). It can be hydrolysed by lysozyme, and the degraded products of chitosan (amino sugars) are also nontoxic, nonimmunogenic and noncarcinogenic, being completely absorbed by the human body (Wilson *et al*., 2010). Chitosan is produced commercially by deacetylation of chitin, naturally occurring polysaccharides which is the structural element in the exoskeleton of crustaceans, it acts as a copolymer of varying amounts of N-acetyl glucosamine and N-glucosamine repeated units (Shi *et al*.,2006) .Tetanus toxoid is a chemically (formaldehyde) detoxified form of the tetanus toxin produced from *Clostridium tetani*. The tetanus neurotoxin is a 1292 amino acid protein consisting of two chains (N-terminal light chain of 52 kDa and C-terminal heavy chain of 98 kDa) linked by a single disulfide bridge (Halpern & Ofthus ,1993). In addition to being a potent antigen, tetanus toxoid has been frequently used as the carrier protein in conjugate vaccines (Astronomo & Burton,2010; Sigurdardottir *et al*.,1997), adding much greater efficacy to carbohydrate vaccines such as from *Streptococcus pneumoniae* (Harding *et al*.,2012). CD25 is also called Interleukin 2 Receptor Alpha .It is a type I transmembrane protein present on activated T cells, activated B cells, some thymocytes, myeloid precursors, and oligodendrocytes that associates with CD122 to form a heterodimer that can act as a high-affinity receptor for IL-2. Though CD25 has been used as a marker to identify CD4+FoxP3+ regulatory T cells in mice, it has been found that a large proportion of resting memory T cells constitutively express CD25 in humans (Triplett *et al*.,2012) . CD25+CD4+ T cells were named regulatory T cells (Treg) and since then have been intensively characterized by many groups (Shevach ,2002; Sakaguchi ,2004). CD29 is the integrin beta1 subunit. Integrin family members are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, tissue repair and immune response (Wu *etal*.,2010). Porcine integrin β1 subunit is involved in rejection of pig-to-human tissue xenografts as target of the natural antibodies present in the human serum. Moreover since CD29 ,as part of the β1 integrins very late antigen 4 (VLA-4) and VLA-6 , is involved in homing and differentiation of haematopoietic progenitor cells (Jimenez-marin *et al*.,2000). CD29 play role in the cell adhesion and recognition in immune response.
The aim of the study: Determination of CD25 &CD29 gene expression in albino rats, immunized by Vi antigen of *S.typhi* loaded on chitosan nanoparticles & tetanus toxoid.

MATERIALS AND METHODS

Collection of Samples: All bacterial isolates were obtained from Central Health Laboratory / Baghdad.

Laboratory animals: Twenty -five Albino male rats (*Rattus norvegicus*) were supplied by the College of Veterinary Medicine in AL- Qadisiyah University. Their ages at the start of the experiments were 6-8 weeks. They were divided into five groups; each group contain 5 rats was kept in a separate plastic cage.

Primers: The primers were used in this study, **GAPDH** (glyceraldehyde-3-phosphate dehydrogenase) gene primers used as Housekeeping gene, **CD25** gene and **CD29** gene primers used as target genes for gene expression . These primers were designed by using NCBI- Gene Bank data base and Primer 3 design online, the primers used in quantification of gene

expression using qRT-PCR techniques based SYBER Green DNA binding dye, and supplied by (Bioneer, Korea). As in (Table -1)

Table-1: The Primers, sequences, gene bank accession number, and references

Primer	Sequence		Reference
GAPDH	F	ATGGGAGTTGCTGTTGAAGTCA	(Hayase *et al*., 2005)
	R	CCGAGGGCCCACTAAAGG	
CD25	F	AACGGCACCATCCTAAACTG	This in study
	R	TGTGCACTGACAGTTGTTGC	
CD29	F	AAGCTCACGTGCATGTTGTG	This in study
	R	TTCCAAATCAGCAGCAAGGC	

Vi- capsular polysaccharide Antigen extraction , purification and quantification:

Preparation of bacterial dry weight : according to (Silipo *et al*., 2002**),** Preparation of Vi-antigen: according to (Espositol &Feeley ,1970**)** and the amount of carbohydrates in antigen was estimated by using the method of phenol - sulfuric acid according to (Dubois *et al*.,1956).

Preparation of Chitosan Nanoparticle: Chitosan Nanoparticle supplied by (Sigma USA) and Prepared according to (Huang *et al*., 2008).

Loading antigen on Chitosan Nanoparticles: was antigen loaded on Chitosan nanoparticles according to (Lubben *et al*., 2002).

Safety test: This test was done according to (Guzman *et al*., 2006)to assist the safety of the prepared immunogen.

Sterility test: This test was done according to(Guzman *et al*., 2006)to evaluate the sterility of prepared immunogen.

Challenge Test: this test was done as illustrated in (Tables – 2).

Table -2: The design of the challenge test.

Group	No. of animals	Treatment		
		First dose	**Boosting dose**	**Challenge dose**
		First week	**Third week**	**Fifth week**
T1	5	20+21.8µg/ 0.25ml	20+21.8µg/ 0.25ml	40+43.75µg/0. 5ml
T2	5	20µg/0.25ml	20µg/0.25ml	40µg/0.5ml
T3	5	20+20µg/ 0.25ml	20+20µg/ 0.25ml	40+40µg/0. 5ml
T4	5	20+20+21.8µg/ 0.25ml	20+20+21.8µg/ 0.25ml	40+40+43.75 µg/ 0. 5ml
C	5	0.25ml(PBS)	0.25ml(PBS)	0. 5ml(PBS)

C: control. T1: ,Vi Ag + Ch. , T2: Vi Ag , T3: Vi Ag+ TT and T4: Vi Ag+ Ch. + TT

Blood samples collection: After 7 days from the last dose of treatment, 3ml of blood samples were collected from rats via intracardiac puncture under general anesthesia by using

diethylether the collected blood samples were divided into two portions; first was treated with anticoagulant was used for estimation the total and differential of WBC count as well as Real-Time PCR. The second portion left until clotting then centrifuge of supernatant to separate the serum and then transferred to suitable plane tube for serological tests (Lewis *et al*., 2001).

Quantitative Reverse Transcription Real-Time PCR(RT-qPCR): Quantitative Reveres Transcription Real-Time PCR technique was performed for estimation of relative quantification (gene expression analysis of CD25&CD29). This technique was done according to method described by (Wang &Hardy ,2004). the following thermocycler protocol in the following (Table -3):

Table -3: thermocycler protocol

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	20 sec	45
Annealing\Extention Detection(scan)	60 °C	30 sec	
Melting	60-95°C	0.5 sec	1

Data analysis of qRT-PCR: The data results of qRT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method that described by (Livak &Schmittgen ,2001).

Passive haemagglutination test: this test was done according to (Boyden,1951)in an attempt to evaluate the antibody titer.

Total leukocytes count : was done according to (Haen ,1995).

Histopathological Study: histological slices were taken from the animals used in experiments for thymus gland & spleen (Allen &Cameron,2004).

Statistical Analysis: They data were statistically analyzed using the statistical package SPSS (Statistical Package for Social Sciences) version 10.0 for windows. The investigated parameters were presented in as mean ± standard error (S.E.), and differences between means were assessed by ANOVA (analysis of variance), followed by LSD (least significant difference). The difference was considered significant when the probability (P) value was ≤ 0.05 (McDonald , 2009).

RESULTS

Results showed a significant increase ($P\leq 0.05$) in antibody titer of all groups in comparison to control group as shown in (Table-4).
Also results showed a significant increase ($P\leq 0.05$) in Total white blood count of all groups in comparison to control group. as shown in (Table -5).

Table-4: Antibody titers of *S.typhi*

Croup	Mean ± SE
c	0 ± 0^{b}
T1	620.8 ± 364.8^{a}
T2	448 ± 117.73^{ab}
T3	537.6 ± 142.53^{ab}
T4	121.6 ± 38.4^{ab}

Different letters refers to significant differences between groups ($P\leq0.05$).

Table -5: Total WBCs counts

Croup	Mean ± SE
C	5500 ± 230.94^{a}
T1	21020 ± 891.85^{c}
T2	9980 ± 572.18^{b}
T3	10740 ± 980.61^{b}
T4	10650 ± 1217.78^{b}

Histopathological findings indicated: a remarkable activity in lymphoid nodes & thymus gland included the enlargement of sizes of lymph nodes in addition to increased numbers of the active nodes as show in (Figure-1).

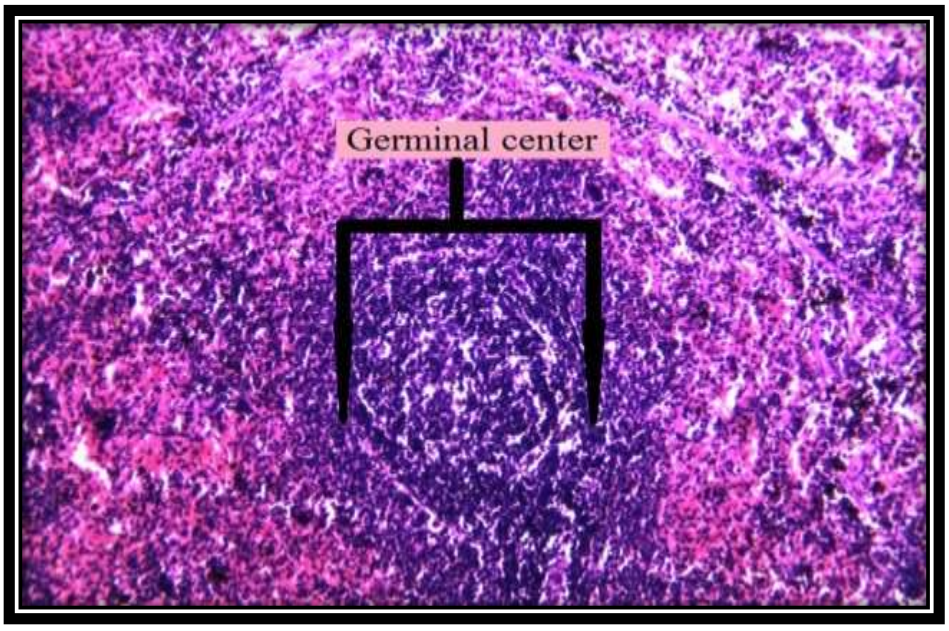

Figure-1: increased number of the active lymph nodes also enlargement of size of these nodes.

Quantitative Reverse Transcriptase Real- Time PCR: Quantitative Reveres Transcription Real-Time PCR (RT-qPCR) was performed for measurement of relative quantification (gene expression analysis) for CD25 and CD29 genes expression levels normalized by housekeeping gene expression (GAPDH).RT-qPCR quantification method in Real-Time PCR system was dependent on the values threshold cycle numbers (CT) of amplification plot of target genes and housekeeping gene. Where the result of Real-Time PCR amplification plot of housekeeping gene GAPDH gene appeared no difference in CT value, where the control group (CT=27) while the treated groups which also appeared (CT=27) (Figure-2) While, The results of Real-Time PCR amplification plot of target genes (CD25 and CD29) showed differences in CT value between control and treatment groups (Figure-3) and (Figure-4)

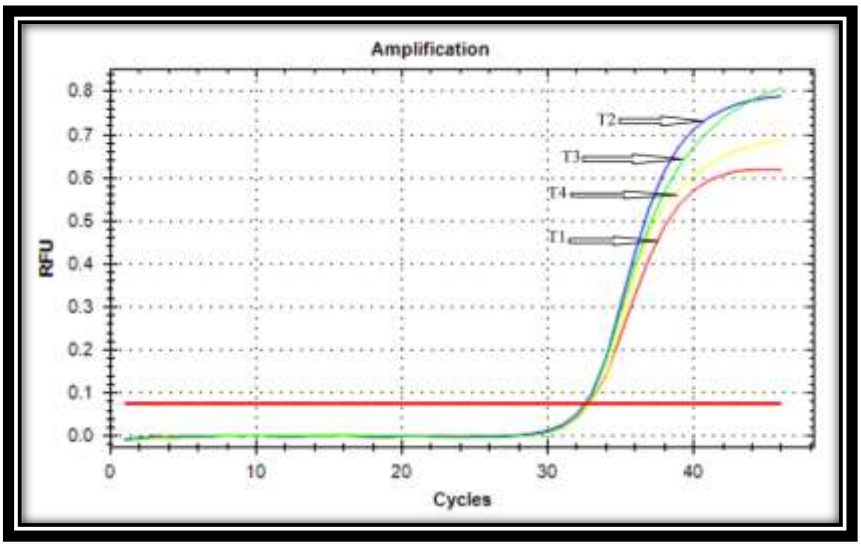

Figure -2: Amplification plot GAPDH gene of RT-qPCR

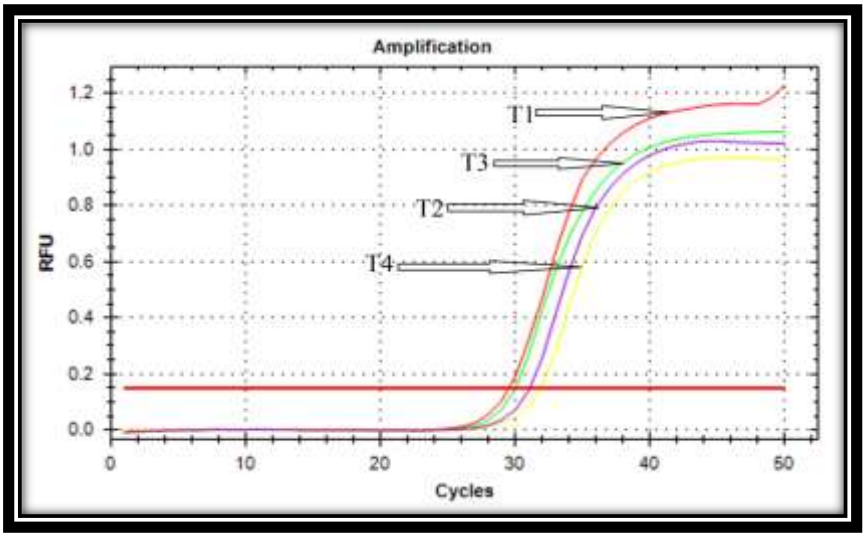

Figure -3: Amplification plot CD25 gene of RT-qPCR

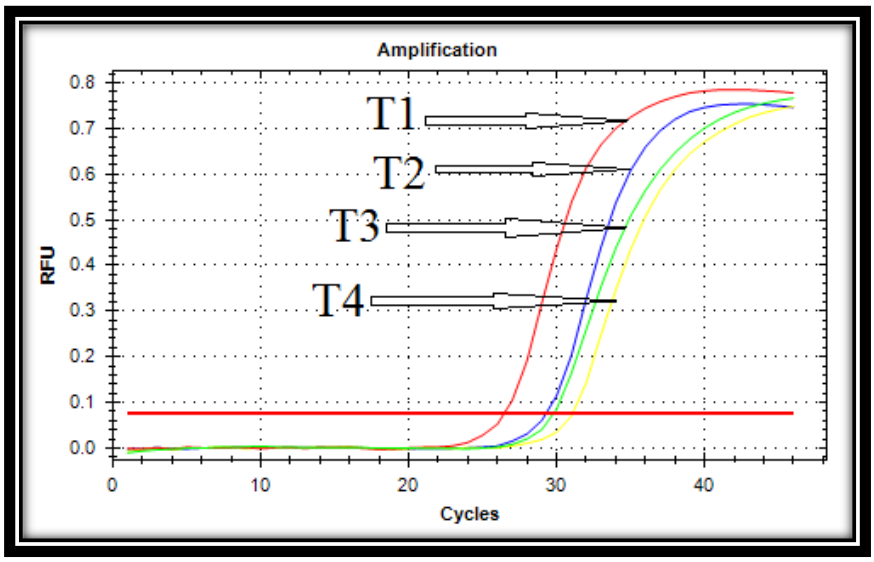

Figure -4: Amplification plot CD29 gene of RT-qPCR

Relative gene expression: The relative expression of target genes (CD25 & CD29) in rats blood was calculated by using Livak Method (2^-ΔΔCT) that dependent on normalization of RT-qPCR (CT values) of target genes with housekeeping gene (GAPDH) as reference gene in control and treatment groups. The results of relative gene expression in CD25 gene which showed clear difference in fold change of gene expression levels between control and treatment groups. Where, T1 group was showed down regulated (2.5604±0.64), T2 group was recorded up regulation at (3.7032±0.54), T3 group was documented down regulation at (4.4739±1), T4 group was appeared up regulation at (5.1936±2.17) relative to control groups that is equal to 1 fold change of gene expression levels as in and (figure-5).The statistical analysis of relative gene expression in CD25 gene was found significant differences in treatment groups compared with control groups at level ($P \leq 0.05$).

The results of relative gene expression in CD29 gene which showed clear difference in fold change of gene expression levels between control and treatment groups. Where, T1 group was appeared down regulation at (33.8276±16.6), T2 group was appeared up regulation at (59.6817±26.65), T3 group was showed down regulation at (162.3256±89.52), T4 group was appeared up regulation at (37.9605±3.09) relative to control groups that is equal to 1 fold change of gene expression levels as in (figure-6).The statistical analysis of relative gene expression in CD29 gene was found significant differences in treatment groups compared with control groups at level ($P \leq 0.05$) .

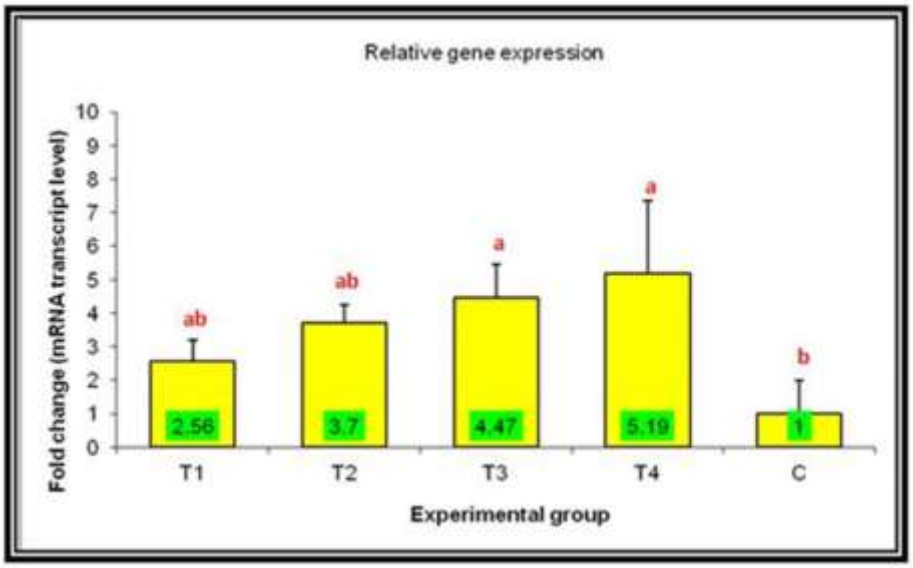

Figure-5: gene expression of CD25 by $2^{-\Delta\Delta CT}$ Livak method.

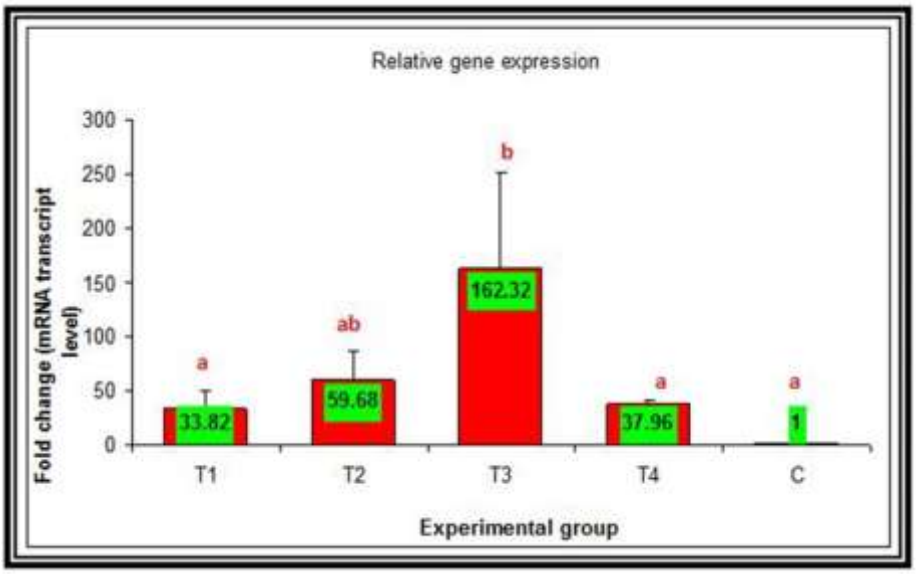

Figure-6: gene expression of CD29 by $2^{-\Delta\Delta CT}$ Livak method.

DISCUSSION

From the obtained results, it was clear that immunization with prepared immunogens led to formation of an important immune response. The elevation in CD25 gene expression may reflect one feature of this response , CD25 is a component of the IL-2 receptor, which is important in T cell proliferation, activation , induction of both T-Reg & T-eff T cells (Brusko *et al*.,2009). It was reported that T-Reg play a major role in regulating of immune responses. Most T-Reg were reported to date have been CD25+ (CD4+CD25+Foxp3+), and it is well established that their induction requires suboptimal stimulation of the T-cell receptor and related cytokines (Horwitz *et al*.,2008). Vi Ag (Vi-CPS) is a successful vaccine alone as reported studies, but its linking with diphtheria toxoid or tetanus toxoid as carrier protein , seems

increasing its immunogenicity & efficiency than used alone (Kothari *et al*.,2014). Recently, this vaccine (Vi +Tetanus Toxoid) is licensed for all ages in India, which starts from over the age of three months. This new generation of typhoid vaccine opens new covenant to prevent and elimination of the disease (Szu ,2013). Chemical nature of Vi antigen reflect that the immune response elicited is mainly T-independent , chitosan nanoparticle seems to be increase the immunogenicity that's may be due to increment of activation chance of B cells which was clearly noticed by elevation of antibody titers in treated groups . As long inflammatory response was observed histologically especially in the activity of lymph proliferation activity of increment of white blood cells.

From the other hand CD29 increased expression may reflect the activity of cell adhesion & recognition in immune response , especially in Tcell & Bcell proliferation. Tetanus toxoid and Vi Ag play important role in immune response and assisted T cell and B cell proliferation and will lead to increase in activation CD29. Human CD4+T cells can be divided into reciprocal memory and naive T cell subsets based on their expression of CD45 isoforms and CD29/integrin beta1 subunit (Kobayashi *et al*., 2004). Chitosan nanoparticle play an important role in increase activation CD29.Chitosan nanoparticle also significantly promoted the production of Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL-5, IL-9 and IL-13) cytokines and CD29 play role in the cell adhesion (Wu *et al*.,2006). Chitosan not only acts as an adjuvant and activates the immune system, but also increases antigen size, and activates the immune system more strongly by its ability to produce nanoparticles, this indicates that it would be a good substitute for a simple antigenic vaccine (purified or recombinant) (Mohammad *et al*.,2011)and also showed remarkable activity in lymphoid nodes and thymus gland included the enlargement of sizes of lymph nodes in addition to increased numbers of the active these nodes. This occur due to the ability of Vi Ag antigens with chitosan nanoparticles that induce Th-1 cells to induce inflammatory phagocytic cells chemically, including macrophages, lymphocytes and neutrophils that leading to enlargement and increment of numbers of the lymph nodes in spleen. These results agreed with researcher who mentioned that showed widening of the white pulp and enlargement of the lymph nodes (Ahmad ,2011).

CONCLUSION

Using of each Chitosan nanoparticles and tetanus toxoid as carriers for Vi antigen that increasing CD25 and CD29 that role in immune response . Using of Vi antigen , chitosan and tetanus toxoid induce humoral and cellular immune response in rats such as elevation of antibody titer, WBCs.

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