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Calcium nanoparticles associated immuno - modulation in wistar rats

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Abstract

Calcium forms the important mineral component of the living system and any alterations in the calcium level may predispose to variety of conditions. Therefore, proper level of calcium in body is thus to be maintained. Being important mineral composition of the body, ~~the~~ various aspects of calcium has been characterized. Relatively, low irritation unlike other nanomaterials, calcium has now been used in nanotechnology. Literature with respect to immunotoxic and cytotoxic effect of calcium nanoparticles is not much cited. However, nanoparticles are known for their smaller size and higher reactivity. The effect of calcium nanoparticle on immunity was taken into focus in the present study. Wistar rats of either sex of 6 weeks age were divided into two groups of control and treatment and nanocalcium was administered daily for a period of 90 days by oral gavaging. Immunopathological alterations occurred/ encountered due to nanoparticle administration were recorded and the correlation was obtained on calcium nanoparticle being immunotoxic or non-immunotoxic. Various parameters of evaluation was involved, Total Leucocyte Count (TLC), Absolute Leucocyte Count (ALC), Serum Globulin, Serum gamma globulin, Lymphocyte Stimulation Test (LST), Macrophage function test (MFT) and Enzyme Linked Immunosorbent Assay (ELISA). The alteration in the level of T-cell and B-cell population was evident of the immunotoxic and cytotoxic effect of calcium nanoparticles. But still much more research into the aspect is needed. Calcium nanoparticles like other metal nanoparticles have resulted in some of the similar results in the present study. As calcium nanoparticles are being used in gene therapy, drug delivery, vaccine adjuvants and many more, henceforth, immunopathological alteration due to calcium nanoparticles might help in knowing the varied aspects of calcium *in-vivo.*

Key words: Calcium nanoparticles, Immunopathology, T and B-cells decrease.

Introduction

Nanoparticles are particles with measurements in the range between 1-100 nm (Laurent *et al*., 2008). With relevant research, incalculable meaning of nanoparticles has been expressed, which says that nanoparticles need the entirety of its measurements to be in the scope of nanoscale (ISO Standards-TC229). However, the European Commission (Europa Commission, 2011) adjusted the prior meaning of nano-particles and characterized that a nano-object needs just one of its measurements in the scope of nano-scale. Nano-particles can be distinctively arranged as: (1) *Ecological*, produced from backwoods flames and remains; (2) *Non-engineered*, speak to side-effects of human action including power plants and incinerators; and (3) *Engineered*, Engineered nanoparticles are refined from mass materials to offer phenomenal collaborations (Aggarwal *et al*., 2009). Engineered materials can be blended as unadulterated particles or composites and in different shapes and sizes just as surface highlights which can moreover be conjugated to various bioactive atoms, making a practically limitless number of varieties with a boundless potential for organic applications (Kawai *et al*., 2011).

Engineered nanoparticles have created a potential impact on various fields like medicine, immunology, cardiology, endocrinology, ophthalmology, oncology, etc. (Bhatia, 2016; Shin-Woo Ha, 2013). The nanotechnology is used in various field including, drug delivery, gene therapy, anti-cancer therapy, imaging and diagnosis (Gwinn and Vallyathan, 2006). Nanotechnology applications in veterinary sciences include diagnosis and treatment of disease using biochips, food safety through identity preservation, breeding, and preservation using nanotubes implantation (Patil *et al*., 2009). Various metal nanoparticles (copper, silica, zinc, silver, gold) which are engineered for the above mentioned procedure has been analysed for their toxic and cytotoxic vulnerability on the living system however, studies based on calcium nanoparticles are not much in literature.

Calcium is one of the most significant mineral components of the living framework, as its inadequacy may prompt a large number of deficiency disorders. Detrimental effect may be seen on Vitamin D digestion and subsequently causes osteoporosis, osteomalacia, rickets and fractures. Research studies have discovered calcium effective in treatment of colon

cancer (Bai *et al*., 2017; Urbanska, 2019) and other therapeutic effects (Bisht and Jha, 2017; Dizaj *et al*., 2015; Kawai *et al*., 2011). Calcium being an integral part of the body shows less inflammatory reaction unlike other engineered nanoparticles. Thus, its nanoform has been utilized in biomedical sciences. Nanoform of calcium is presently used in toothpastes, as anti-microbial agent in food (Roy *et al*., 2013) in vaccine administration (He *et al*., 2000), drug delivery and medicines (Sawai and Igarashi, 2002).

Amidst, considerable number of focal points, the hostile impacts of calcium has stayed covered up for very long. Ongoing examinations on calcium have uncovered its effect in tumor cell reprogramming (Senchukova *et al*., 2019; Som *et al*., 2018). Likewise calcium and its relative immunopathology have not been given genuinely necessary consideration. Along these lines, the present paper focuses the immunopathology of calcium nanoparticles. Nanoparticles being smaller in size have a bigger surface volume which expands their reactivity and makes them conceivably toxic. Principle mechanism of nanotoxicity is the generation of reactive oxygen species (ROS) (Gonzalez *et al*., 2008), creation of ROS which at last outcomes into cytotoxicity (Fu *et al*., 2014).

Materials and method

The research was conducted with due approval from the Institutional Animal Ethics Committee (IAEC). The research was conducted using no observed adverse effect level dose (NOAEL) of calcium nanoparticles at the rate of 1000 mg/kg body weight (Sung *et al*., 2015). Preformed white color Nano-calcium powder of 80 nm particle size were procured from the Siesco Research Laboratory, Mumbai, with a molecular weight of 100.09 and 98% assay and a shelf life of 60 days. The nanoparticles were administered through oral gavaging to Wistar rats daily for a period of 90 days. Rats were divided into two groups of calcium control rats (G1 group) and second of nano- calcium treated rats (G2 group). Rats of either sex of 6 weeks age were taken for this purpose. Experiment was planned grouping five rats for each 30 days interval. The blood was collected to measure various hematological and biochemical patterns for alterations due to calcium nanoparticles administration. Various parameters were evaluated including Total leucocyte count (TLC), Absolute lymphocyte count (ALC), Enzyme linked immune-sorbent assay (ELISA), Serum Globulin, Lymphocyte blastogenesis test (LST) and Macrophage function test (MFT). The tests were conducted at every 30-day interval for a period of 90 days.

Lymphocyte blastogenesis assay: For lymphocyte blastogenesis assay, spleen was collected from the nano-calcium treated rats in phosphate buffered saline (PBS). Then the splenocytes were prepared from the spleen by trituration. Supernatant was discarded and the cells were washed in RPMI-1640 (basal culture media). After this the cells were subjected to trypan blue dye for assessing the live and dead lymphocytes. After the live and dead cell count, the cell suspension was prepared as 1 x10^7 cells/ml and then poured into 96 wells plate along with the mitogen. For T- cells, Con-A (5 µg/ml) and PHA (5 µg/ml) mitogens were used and for B- cells LPS (5 µg/ml) was used. Triplicate wells were prepared and incubated in CO_2 incubator for 68 hours. Following the procedure 10 microlitre of MTT dye (5mg/ml) was added to the entire plate and then incubated for 4 hours. Addition of the MTT dye leads to the formation of the formazon crystal and the yellow color of the dye changes to purple. The crystals formed are then diluted using acid isopropanol or DMSO to all the wells and mixed properly. After few minutes the OD was taken at 570 nm in microplate spectrophotometer reader (Mosmann, 1983; Rai-el-Balhaa *et al*., 1985).

***Macrophage function test*:** Macrophage function test evaluates alteration in the concentration and activity of the macrophages upon treatment with calcium nanoparticles. For this purpose, mineral oil was injected peritoneally to induce inflammatory reaction and then the following day the sample was collected intraperitoneally. To the sample collected equal amount of RPMI-1640 was added. In another centrifuge tube equal volume of histopaque 1119 (procured from Hi-media as GranuloSep TM GSM 1119) was poured and sample mixture containing RPMI -1640 was overlaid slowly without mixing the content. Centrifugation of the tube at 400g (10- 15 minute) was done, an opaque layer was formed this opaque layer was collected. This layer is then subjected to nitroblue tetrazolium (NBT) test. A mixture was prepared containing 0.3 ml of 0.2% NBT in PBS, 0.2 ml of cell suspension and 0.1 ml activated plasma (activated plasma is prepared by mixing 1 ml plasma + 15 microlitre of LPS). The mixture was then incubated in water bath at 37°C. The reaction was stopped by adding cold PBS. Centrifugation of cell suspension was done at 500g for 5 minute and supernatant was discarded. The cells were re-suspended in 0.5 ml PBS. Smear from the cell suspension was made and airdried and methanol fixed. Counter stain using 0.5% safranin for 2 minutes was done. Slides were air dried and viewed (Chauhan, 1998).

Serum gamma globulin: For estimation of serum gamma globulin; a solution of ammonium sulfate (19.5%) and sodium chloride (2.03%) was prepared. 5.7 ml of this solution was then put in a centrifuge tube and overlaid with 0.3 ml of clear serum. The mixture was then mixed gently and kept on ice bath for 15 minutes followed by centrifugation of the mixture at 1250g for 10 minutes. Precipitate obtained, was dissolved in 0.2 ml of normal saline solution and process of precipitation was repeated. Finally, all the precipitates were dissolved in 2 ml of normal saline solution. Finally 5 ml of biuret reagent was added. The mixture was then kept at room temperature for 10 minutes and then OD was read at 555nm in UV-Vis spectrophotometer (Chauhan, 1998).

Results

The values obtained over the period of 90 days were recorded and was then assessed for alteration in the hematological and immunological parameters. The recorded data is presented with mean ± SE and statistically analysed with two way analysis of variance (ANOVA) using SPSS software with $p<0.05$.

For hematological alterations, the total leucocyte count was recorded for a period of 90 days. TLC showed decrease in the count in nano-calcium treated rats from 30th to 90th day of treatment (16.19±1.86; 15.05±1.09; 14.87±0.97) as compared with the calcium treated control rats (Table 1 and Fig. 1). The TLC was found to increase in nano-calcium treated rats at 30th day post treatment; however, subsequent decrease in the TLC count was noted after 60th and 90th day post treatment with calcium nanoparticles. Following the decrease in TLC value, decrease in particular cell count was observed as the lymphocyte and neutrophils comprises leucocyte in large numbers. Therefore, absolute lymphocyte count (ALC) was recorded and evaluated for 90 days period. It was observed that ALC showed similar pattern as that of TLC. ALC increased initially at 30th day post treatment (13.62± 1.56); however, a decrease in ALC was noticed both within the group at 60th (12.72± 1.89) and 90th day (12.49± 2.12) post treatment with the calcium nanoparticles. When compared in between the 2 groups, the ALC was found to reduce in nano-calcium treated group (Table 2 and Fig. 2). The absolute neutrophil count (ANC) was estimated for effect on the phagocytic activity due to calcium nanoparticle (Table 3 and Fig. 3). Initially, neutrophil count was increased at 30th day (1.96± 0.28) and then gradual fall in the count was observed at 90th day (1.24±0.33) post nano-calcium treatment

For immunological alteration various tests were employed to record and evaluate the effect of calcium nanoparticles. These include serum globulin, lymphocyte blastogenesis test/ assay (LST), macrophage function test (MFT), hemagglutination inhibition assay (HI) and enzyme linked immune sorbent assay (ELISA).

LST was done to evaluate the effect of calcium nanoparticle on T-cells and B-cells population as they play role in immunity. For this, 3 mitogens were made use of CON-A and PHA- M for T cells and LPS for B-cells, respectively. The value for CON-A was found to decrease from 30th (0.90±0.55) to 90th (0.76± 0.17) day of study with $p<0.05$ at 30th and 60th day of experiment (Table 4 and Fig. 4) and PHA-M was decreased (1.06±0.13; 0.83±0.02; 0.80± 0.11) at 30th, 60th and 90th days of study, respectively, with $p<0.05$ (Table 5 and Fig. 5). LPS (Table 10 and Fig. 10) in calcium nanoparticle treated rats was also found to decrease from 30th (0.79± 0.41) to 90th (0.75± 0.01) day post treatment with calcium nanoparticles. Further, the phagocytic activity was checked with macrophage function test (Table 6 and fig. 6) to access the effect on the phagocytic ability of macrophages. An initial increase in the macrophages was observed at 30th day followed by a slow fall in the count by 90th day post nano-calcium treated rats.

Further, alteration in the B-cell population was checked by serum globulin and serum gamma globulin level. The serum globulin level was found to increase in nano calcium treated rats 2.66± 0.18; 2.85± 0.50; 3.43± 0.27) over the entire study period (Table 7 and Fig. 7). The alteration in immunoglobulin level was checked with increase in gamma globulin levels (Table 8 and Fig. 8). The gamma globulin levels were found to increase in nano-calcium treated rats at 30th (0.68 ±0.06; $p<0.05$), 60th (1.3 ±0.16; $p<0.05$) and 90th (1.32 ± 0.07; $p<0.05$) day post treatment. The HI titer values were used for assessing the effect of calcium nanoparticles on the humoral immunity. The HI titers were increased at 30th (8.2±0.39), 60th (8.2±0.37), and 90th (8.4±0.25) day (Table 9 and Fig.9). However, these alterations were statistically insignificant in between the groups and within the groups. ELISA values were calculated for measuring the effect of calcium nanoparticles on IgG antibodies in serum. The mean ELISA values were also found to increase at 30th, 60th, and 90th, day in between the groups. However, these alterations were insignificant (Table 11 and fig.11). When compared within the group the values were found to increase at 30th, 60th and 90th day (0.923±0.087; 1.216± 0.09; 1.266±0.15) post treatment. The increase in values suggests increased B-cell activation in the peripheral blood lymphocytes. The nano-materials are known to cause increase in lymphocyte production due to ROS generation.

Discussion

The vast majority of the metal-based NP exhibits toxicity through Fenton-type responses and ROS production (Manke *et al*., 2013; Risom *et al*., 2005). Nanoparticles have been accounted to impact intracellular calcium fixations, actuate translation factors, and adjust cytokine creation by means of free radicals (Thannickal and Fanburg, 2000). However, this increase in intracellular calcium or the cytoplasmic calcium due to nanoparticle administration directs the lymphocyte multiplication in the peripheral blood mononuclear cells at initial phase of low dose. Subsequently, higher dose/ prolong use may cause increase in calcium concentration there by activating calcineurin and decreasing the lymphocyte count by activation of TGF β and also due to prolonged use of nanoparticles ROS and nitrogen species are generated hampering the phagocytic cell count gradually. Likewise, calcium nanoparticle also exhibit the effect similar increase in the lymphocyte viz., ALC in the rodents treated with the calcium nanoparticles uncover that calcium additionally acts in the strategies as portrayed for other metal nanoparticles (Manke *et al*., 2013; Petrarca *et al*., 2015).

Nanoparticles other than ROS creation, upon interaction with the biological fluid forms protein corona otherwise called bio-nano interface (Barbero *et al*., 2017; Lundqvist *et al*., 2011; Tenzer *et al*., 2013). The protein corona development decides the association of the nanoparticles (Lynch *et al*., 2007; Mahmoudi *et al*., 2011) and furthermore drags out its flow and in this manner making simple for nanoparticles to incite inflammatory responses through MAPK and NF-κB signaling pathways that control transcription of inflammatory genes such as IL-1β, IL-8, and TNF-α (Borm *et al*., 2006; Hsin *et al*., 2008; Thannickal and Fanburg, 2000). The inflammation causes cascade of response, which prompts increment in the T-cell population. Initially, encounter with the calcium nanoparticles are viewed as remote and this prompts

increment in the immature T-cell population. Many research with other metallic nanoparticles expresses that metallic nanoparticle will in general discharge their metal in its ionic structure which prompts the subsequent harm to the body (Rahman, 2007). In this manner, reaching inference from the examination on different other metallic nanoparticles, calcium whenever discharged in ionic structure (Ca^{++}) will build the grouping of calcium extracellularly. Raised calcium fixation prompts activation of calcineurin, a calcium dependent serine-threonine protein phosphatase. Calcineurin causes resulting increment in transforming growth factor β (TGF-β). Increment in TGF-β elevates ROS and nitrogen oxide creation, which causes mitochondrial damage which diminishes the development of lymphocytes (Zolink *et al*., 2010) and influences insusceptibility. This relates with the possible decrease and altered immune response in the T-cell and B-cell population during LST involving calcium nanoparticle.

Further, the increase in the T-cell population due to inflammation likewise impacts B-cell population indirectly (Mosmann, 1983). The B-cells upon initiation separate into immunoglobulin producing B-cells and along these lines increases the immunoglobulin level in the peripheral blood. Similar outcomes could be found in the current investigation led with calcium nanoparticles as apparent with the rise in HI, ELISA (Brown *et al*., 1990) and serum gamma globulin levels. Besides, the inflammatory response, ROS production due to nanoparticle exposure (Fu *et al*., 2014) may be answerable for damage to visceral organs (Nel *et al*., 2006). The main target organ for the metal nanoparticle is liver and kidney. Nanoparticles damage the mitochondria and the organelle membrane and thus causing cell death due to apoptosis. Any damage to visceral organs may along these lines lead to further alteration in the protein levels in the serum. The ascent in the serum globulin level in rodents treated to calcium nanoparticle may also be attributed to possible damage to visceral organs.

Besides, organelle damage nanoparticles on ROS production likewise cause oxidative stress and instigate arrival of glutathione into its oxidized form glutathione disulfide (Fenoglio *et al*., 2008) thereby enhancing the adverse effect of nanoparticle and causing cell death via apoptosis (Fenoglio *et al*., 2008; Rai-el-Balhaa *et al*., 1985). Similarly, the working of NADPH dependent enzymes is likewise influenced. This NADPH is utilized by the phagocytic cells to direct the procedure of phagocytosis through myeloperoxidase compound. Because of increment in the oxidative pressure and diminishing in the fixation or nonattendance of the NADPH, oxidase hinder the utilization of defensive instrument utilized by neutrophils and macrophages to utilize and change over free radical into hydrogen peroxide particles. Accordingly, it brings about debilitating of the phagocytic system and result in death of influenced macrophages and neutrophils (Eom H and Choi J, 2010; Figueiredo *et al*., 2018; Manke *et al*., 2013; Sanfins *et al*., 2018). At first, the low concentration of the nanoparticles builds the creation of neutrophils and macrophages however; with the ascent in convergence of the nanoparticles the system is hindered there by influencing the insusceptibility (Roy *et al*., 2013; Sanfins *et al*., 2018). The results observed in MFT and ANC might clarify and bolster the mechanism of activity by which the calcium nanoparticle incites the alteration in the resistant arrangement of the nanoparticles treated Wistar rats.

The nanoparticles however, at a low dose improves the immune response as they have been able to elicit the response through inflammatory receptors, increase in neutrophils and macrophages and immunoglobulin levels at initial phase of the study with nano-calcium treatment. However, continued with the same dose of nanoparticle administration the increase obtained in some of the parameters showed alteration viz., lymphocyte neutrophil and macrophage count. Decrease in the level of T-cell and B-cell population was noticed. Thus, for prolong use of nanoparticle in certain biomedical conditions as in oncology, gene therapy, implantation etc., the effect of nanoparticles should be assessed. Also the cytotoxic and immunotoxic effect regarding calcium nanoparticle are not much cited in literature and hence more information in this regard is indeed necessary.

Conclusion

Nanoparticle owing to its smaller size and larger surface area is finding its use in biomedical sciences. Nanoparticle has been evaluated for their role in drug delivery, vaccine adjuvants and others. Nanoparticles has also been categorized into various types. Engineered nanoparticles commonly used are the metal or metal oxide nanoparticles in biomedical sciences and has uniform size with range of 1- 100 nm. Recent research in engineered nanoparticles have revealed their cytotoxic and immunotoxic activity. Recently, calcium nanoparticles are in use because of being an integral component of the living system and induce less inflammatory reaction upon administration. Calcium has also replaced aluminum nanoparticles as adjuvants in vaccine and is also being used in drug-targeted therapy.

Further, effect of nanoparticle varies depending on the size and shape and the purpose they are used for in biomedical sciences. The effect of a particular nanoparticle may differ at different sizes. A nanoparticle with a size of 10 nm will have more pronounced cytotoxic and immunotoxic effect as compared to the same nanoparticle at 30 nm or at 50 nm range. Therefore, the toxic effect of nanoparticles should be measured at particular range for which they are to be used.

Calcium being the internal component of the body has shown its potential to be used over other nanoparticles because of the less inflammatory effects. However, much literature and evidence based study for the toxic effect of the calcium is not present. Thus, research on various size of calcium nano-form is indeed needed. Therefore, the present study was conducted to know the extent of damage caused by nano-calcium. Unlike, other metal nanoparticles, the mechanism of toxicity of calcium is also similar, however, more evidence are needed

on role of calcium nanoparticles on living system. Calcium nanoparticles like other metal nanoparticles have resulted in some of the similar results in the present study. These details inferred however needs more similar facts on the effect of calcium nanoparticles. Also, metal nanoparticles are known to activate carcinogenesis and recent study has cited the possible role of calcium in causing cancer reprogramming. Therefore, much insight into the mechanism and functioning of the calcium nanoparticles need to be evaluated.

Conflict of interests

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Table 1: Mean Total Leucocyte Count (10^3/ microlitre) in different groups of experimental rats at different time intervals of the experimental study (Mean ± SE)

DPT	Mean Total Leucocyte Count (Mean ± SE) in 10^3/ microlitre	
	Group 1	Group 2
0 day	12.52± 2.02	12.52± 2.02
30 day	15.24± 2.15	16.19± 1.86 (4.18%)
60 day	15.28 ± 1.74	15.05± 1.09 (-1.5%)
90 day	15.11±2.52	14.87±0.97 (-1.39%)

Table 2: Absolute lymphocyte count (10^3/ microlitre) in different groups of experimental rats at different time intervals of the experimental study (Mean ± SE)

DPT	Absolute lymphocyte count (Mean ± SE) in10^3/ microliter	
	Group 1	Group 2
0 day	10.25± 1.74	10.25±1.74
30 day	11.93±1.28	13.62± 1.56 (14.16%)
60 day	11.69± 0.88	12.72± 1.89 (8.81%)
90 day	11.69± 0.52	12.49± 2.12 (6.84%)

Table 3: Absolute Neutrophil Count (10^3/ microlitre) in different groups of experimental rats at different time intervals of the experimental study (Mean ± SE)

DPT	Absolute neutrophil count (Mean ± SE) in10^3/ microlitre	
	Group 1	Group 2
0 day	1.08± 0.20	1.08± 0.20
30 day	1.12± 1.13 (18.58%)	1.96± 0.28 (75%)
60 day	1.2± 0.77 (19.13%)	1.67± 0.73 (39%)
90 day	1.15± 0.58 (73.39%)	1.24±0.33 (7.8%)

Table 4: Mean T lymphocyte blastogenesis assay using Con-A mitogen at different time intervals in experimental rats (Mean± SE)

DPT	Mean T-lymphocyte blastogenesis assay (Mean± SE) in ΔOD	
	Group 1	Group 2
30 day	$0.75±0.11^{a}$	$0.90±0.55^{a}$ (20%)
60 day	$0.75±0.11^{a}$	$0.91± 0.66^{a}$ (21.33%)
90 day	0.76±0.01	0.76± 0.17 (0%)

Table 5: Mean T lymphocyte blastogenesis assay using PHA-M mitogen at different time intervals in experimental rats (Mean± SE)

DPT	Mean T-lymphocyte blastogenesis assay using PHA-M (Mean± SE) in ΔOD	
	Group 1	Group 2
30 day	$0.74±0.08^{Bb}$	$1.06±0.13^{Aa}$ (43.24%)
60 day	$0.74±0.05^{AB}$	$0.83±0.02^{B}$ (12.16%)
90 day	$0.78±0.18^{A}$	$0.80± 0.11^{B}$ (2.56%)

Table 6: Macrophage function test, NBT positive cells (%) on pooled blood samples in different groups of experimental rats at different time intervals of the experimental study

DPT	NBT positive cells (%)	
	Group 1	Group 2
0 day	38	38
30 day	36	44 (22.2%)
60 day	40	44 (10%)
90 day	37	40 (8%)

Table 7: Serum globulin concentration (g/dl) in different groups of experimental rats at different time intervals of the experimental study (Mean ± SE)

DPT	Mean total globulin concentration (Mean ± SE) in g/dl	
	Group 1	Group 2
0 day	2.62± 0.40	2.62± 0.40
30 day	$2.63± 0.11^{B}$	$2.66± 0.18^{B}$ (1.14%)
60 day	$2.69± 0.27^{Aa}$	$2.85± 0.50^{Bb}$ (5.95%)
90 day	$2.68± 0.62^{A}$	$3.43± 0.27^{A}$ (27.99%)

Table 8: Serum gamma globulin (g/dl) in different groups of experimental rats at different time intervals of the experimental study (Mean ± SE)

DPT	Mean serum gamma globulin (Mean ± SE) in g/dl	
	Group 1	Group 2
0 day	0.47±0.07	0.47±0.07
30 day	$0.38 ±0.03^{Bb}$	$0.68 ±0.06^{Ba}$ (78.94%)
60 day	$0.78 ±0.08^{Ab}$	$1.3 ±0.16^{Aa}$ (66.67%)
90 day	$0.85 ±0.09^{Ab}$	$1.318 ± 0.07^{Aa}$ (55.98%)

Table 9: Mean hemagglutination inhibition (HI) titre (log_2) in different groups of experimental rats at different time intervals (Mean±SE)

DPT	Mean hemagglutination inhibition (HI) titre (Mean±SE) values in log_2	
	Group 1	Group 2
30 day	7.8±0.66 (-140%)	8.2±0.39 (5.13%)
60 day	7.4±0.25 (20%)	8.2±0.37 (10.8%)
90 day	7.6±0.40 (-80%)	8.4±0.25 (10.5%)

Table 10: Mean B- lymphocyte blastogenesis assay in rats using LPS mitogen at different time interval (Mean± SE)

DPT	Mean ΔOD of B- lymphocyte blastogenesis assay (Mean± SE)	
	Group 1	Group 2
30 day	0.83± 0.89	0.79± 0.41 (-4.82%)
60 day	0.82± 0.01	0.78± 0.02 (-4.88%)
90 day	0.83± 0.46	0.75± 0.01 (-5.06%)

Table 11: Mean ELISA values in different groups of experimental rats at different time intervals (Mean ± SE)

DPT	Mean ELISA values (Mean± SE) in ΔOD	
	Group 1	Group 2
30 day	0.952±0.07	0.923±0.087 (-4.94%)
60 day	1.099±0.04	1.216± 0.09 (10.65%)
90 day	1.097±0.13	1.266±0.15 (13.35%)

Fig. 1: Mean Total Leucocyte Count (TLC, 10^3/microlitre) in different groups of experimental rats at different time intervals of the experimental study

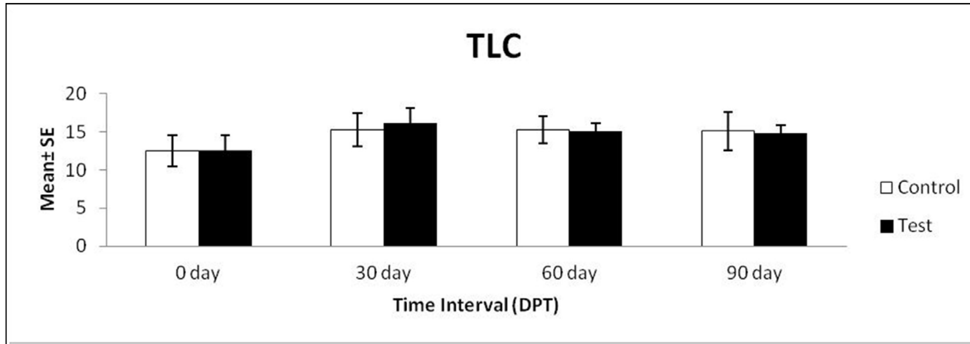

Fig. 2: Mean absolute lymphocyte count (ALC, 10^3/µl) in different groups of experimental rats at different time intervals of the experimental study.

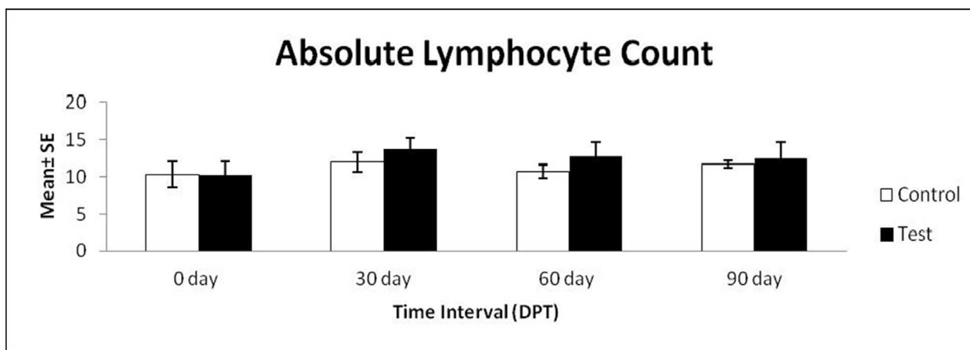

Fig. 3: Mean absolute lymphocyte count (ALC, 10^3/µl) in different groups of experimental rats at different time intervals of the experimental study.

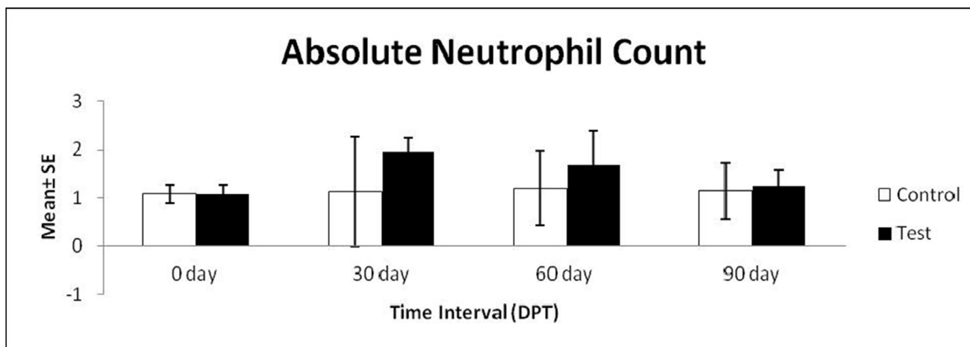

Fig.4: T- lymphocyte blastogenesis in different group of rats using Con-A mitogen at different time intervals of the experimental study

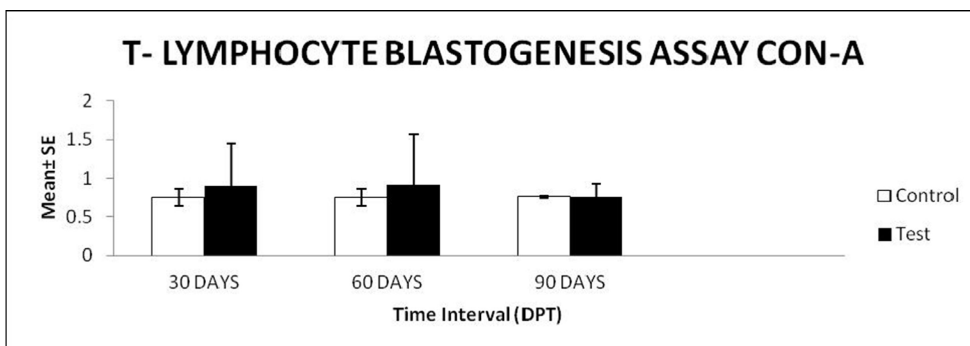

Fig. 5: T- lymphocyte blastogenesis in different group of rats using PHA-M mitogen at different time intervals of the experimental study

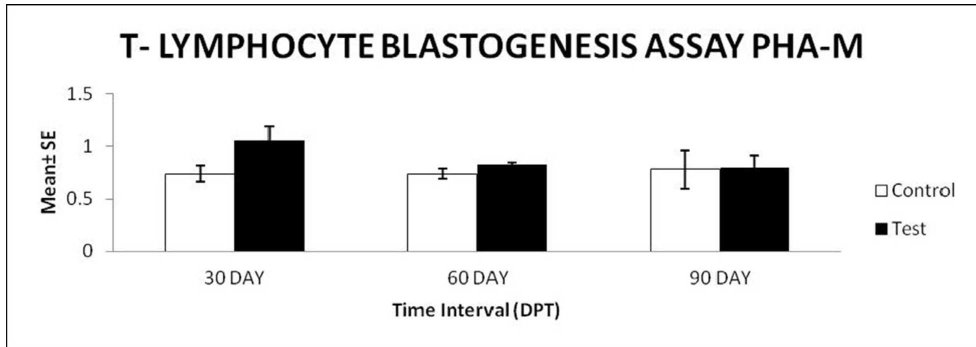

Fig.6: NBT positive cells (%) on pooled blood samples in different groups of experimental rats at different time intervals of the experimental study

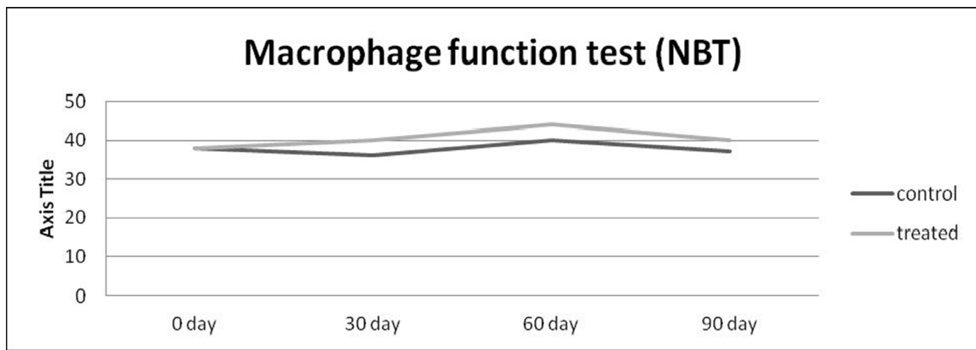

Fig. 7: Mean Serum globulin (g/dl) in different groups of experimental rats at different time intervals of the experimental study

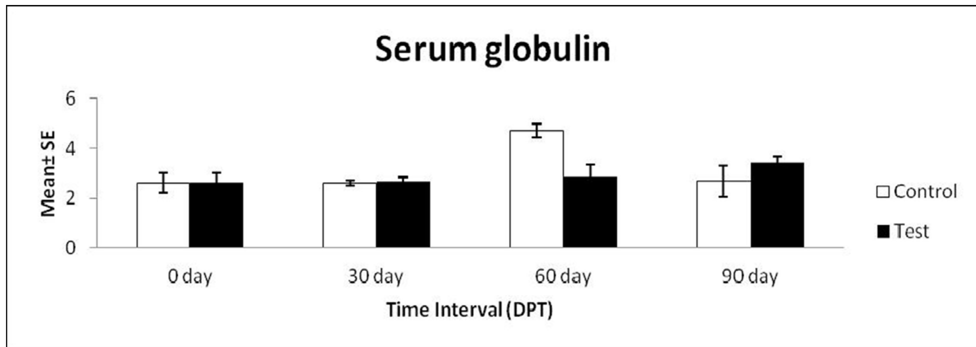

Fig.8: Mean Serum gamma globulin (g/dl) in different groups of experimental rats at different time intervals of the experimental study

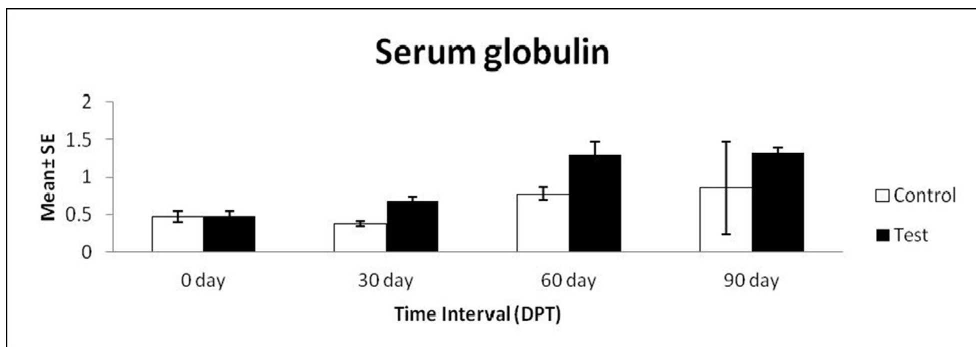

Fig.9: Mean hemagglutination inhibition (HI) titre (log_2) in different groups of experimental rats at different time intervals

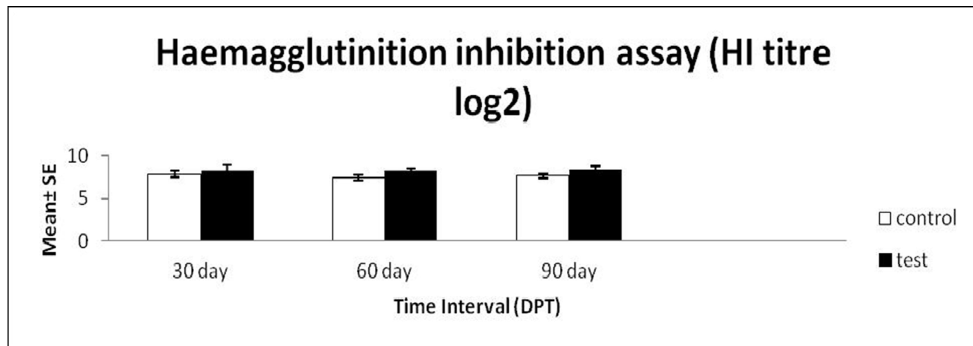

Fig.10 B- lymphocyte blastogenesis in different group of rats at different time intervals of the experimental study

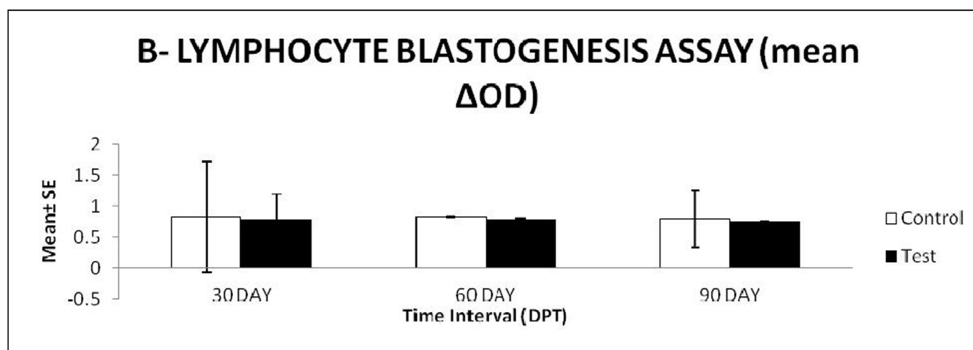

Fig: 11: Mean ELISA values in different group of rats at different time intervals of the experimental study at 90 days.