The role of zinc on anti-newcastle disease virus specific antibody response and agranulocytes count in rabbits treated with methotrexate and prednisolone

Muhammad Khalid Tipu*[1,4], Uzma Saleem[2], Khalid Hussain[2], Khushi Muhammad[3], Furqan Khurshid Hashmi[2], Muhammad Islam[1] and Bashir Ahmad[2]

[1]Lahore College of Pharmaceutical Sciences, 18-Km Raiwind Road, Lahore, Pakistan
[2]University College of Pharmacy, University of the Punjab, Lahore, Pakistan
[3]Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[4]Department of Pharmacy, Quaid-i-Azam University, Islamabad, Pakistan

Abstract: Zinc (Zn) plays a pivotal role in highly proliferative tissues including immune system. The long-term therapy of neoplastic and autoimmune disorders is associated with immunosuppression and myleosuppression. In the current study role of Zn on anti-Newcastle disease virus response and agranulocytes count of methotrexate and prednisolone treated rabbits. Thirty six healthy rabbits were randomly segregated into six groups (group I to VI) each containing six rabbits. Oil based Newcastle disease virus (NDV) vaccine was administered subcutaneously to rabbits of all the groups at day 0 and 21 and after one week, all the groups received Zn, (Zn + prednisolone), prednisolone, (Zn + methotrexate) methotrexate orally from day 7 to day 21, except the control. The serum antibody titer, total and differential leukocyte count were measured weekly for 6 weeks. The administration of zinc in combination with methotrexate showed same antibody titer as that of the control suggesting that Zn has ability to counteract the methotrexate-induced immunosuppression. However, Zn did not show any significant impact in combination with prednisolone ($p<0.05$). The results of the present study indicate that co-administration of Zn and methotrexate is beneficial in the activity of immune system.

Keywords: Immunosuppression; methotrexate, prednisolone; antibody titer.

INTRODUCTION

Zn is essential trace element for growth and maintenance of normal health. It affects expression of many genes in the cells of immune system (Haase *et al.,* 2007). Moreover it also has very important role in regulation of apoptosis, which is very important for killing of autoimmune cells, infected cells and tumor cells by cytotoxic T-cells or NK-cells (Wellinghausen and Rink, 1998). The monocytes are the cells, involved in both specific and non-specific immune response and differentiate into macrophages in tissues. Apart from their phagocytic function, macrophages not only act as antigen presenting cell but also release cytokines for activation of other immune cells. Theses action involves the Toll-like receptor stimulated by antigen e.g. lipopolysaccharides. An optimum level of Zn is essential for the release of proinflammatory cytokines and activation of antigen presenting cells (Haase and Rink, 2007). It is also required for optimum activity of thymulin (a hormone of thymus), which induces several T-cell markers of differentiation and promoting cytotoxic suppressor function and production of interleukin-2 (IL-2) (Prasad, 1988). Zn is needed for the maintenance of a ratio of T-helper $cell_1$ and T-helper $cell_2$ ($Th_1 > Th_2$). Th_1 cells are involved in macrophage activation, complement fixation and antibody based opsonization (Prasad, 2008). Zn enhances proliferation ability of B-cells, pre-B cell and immature B cell (Ibs and Lothar, 2003). Secondly supplementation with Zn also improve the intestinal damage (Tran *et al*., 2003) and permeabilitydisorder (Zhang and Guo, 2009).

Methotrexate is an agent possessing immunosuppressant, anti-rheumatic and anti-neoplastic properties. It inhibits dihydrofolate reductase, increases adenosine release, inhibits adenosine deaminase and inhibits neutrophil chemotaxis (Chandra, 1984). It improves production of IL-2 by an effect on polyamine synthesis and decreases production of IL-1 and secretion of IL-6 (Cronstein and Memill, 1996). It is used in treatment of neoplastic disorder like leukemia, ostegenic sarcoma and non-neoplastic disorder e.g. rheumatoid arthritis and psoriasis (Neurath *et al.,* 1999). Similarly, prednisolone is a synthetic glucocorticoids, which exerts a suppressive action on each stage of immune response (Zurier & Weissman, 1973). It inhibits synthesis of various cytokines by cells of the immune system i.e. IL-1, IL-2,IL-3, IL-6, IL-8 and tumor necrotic factor (TNF) alpha, which are critical in generation of immune response (Bernard and Keith, 2001).

Immunosuppression is one of the side effects of methotrexate and prednisolone. Lifelong therapy of these

**Corresponding author:* e-mail: tippupharma@yahoo.co.uk

drugs is essential for maintaining daily routine life of the patients but may result in suppression of their immune system and bone marrow. Such immunocompromised patients become susceptible to many infectious agents which adversely effects the quality of life and increase cost of the treatment (Corti *et el.,* 2009 and Fishman, 2011). Moreover other adverse effects also enhance the agony of disease (Verstappen *et al*., 2010).

Therefore corrective effects of Zn may be exploited to reduce the immunosuppression associated with methotrexate and prednisolone therapy. Therefore, current study was designed to measure the antibody titer and agranulocytes count in drug treated rabbits.

MATERIAL AND METHODS

Experimental animals

The study was carried out on 3 months aged thirty six healthy rabbits of either sex. The weight of the rabbits varied from 1.2-1.5 kg. The rabbits were obtained from Animal House of University College of Pharmacy, University of Punjab, and Lahore and were maintained in animal cages of sufficient space under controlled conditions of temperature, humidity and 12 hours light/dark cycle throughout the period of study. The green fodder was provided at rate of one kg/rabbit and fresh water was given ad lib.

Design of the study

Animals were randomly segregated into six groups each having six animals. Zn in dose of 1mg/kg (Philip & Samantha,2002), prednisolone in dose of 1.2 mg/kg (Godeau *et al*., 1994) and methotrexate in dose of 20 mg/mm^2 (Frank *et al*., 1998) were given to animals segregated into group I (Zn), group II (prednisolone), group III (methotrexate), group IV (Zn + prednisolone), group V (Zn + methotrexate) from day 7 to 21 whilst group VI acted as a control.

Immunization model

Immunization model reported previously was applied with slight modification was used (Postic *et al.,* 1966). Oil based Newcastle disease virus (NDV) vaccine was used as immunogen. One milliliter of NDV vaccine was injected to each animal subcutaneously at day 0 and 21 of the study. Three milliliters of blood was collected from marginal ear vein on weekly basis for analytical purposes from day 0 to 42. The serum from each sample was separated and stored in properly labeled vials at -40^{o}C till required for further processing.

Monitoring of Anti-NDV antibodies and agranulocytes

Antibody titer of each serum sample against Newcastle Disease virus was determined using hemagglutination inhibition (HI) test (Allen *et al.*, 1974). The end dilution points of serological test were recorded by well-number. The geometric mean titre was calculated from a table (Burgh, 1978). Total leukocyte, monocyte and lymphocytes were measured by standard method as described in manual (Ghai, 2007).

RESULTS

The results of anti-NDV hemagglutination inhibiting (HI) antibody titer of the rabbits that have been primed at day 0 and boosted on day 21 are shown in Table 1. All the treatment groups showed detectable levels of the antibody titre on day 7 reflecting the mobilization of immune system. The level of immunogenicity was same for all the groups. The same result were recorded at day 14 ($p=0.155$) but higher antibody level than the day 0. On day 21 antibody titer was found to be continually increasing, however, all the treatments showed insignificant difference ($p=0.229$). On day 28 (7 days after booster dose) there was a significant rise in the antibody titer and different treatments showed different antibody levels ($p=0.000$). The maximum titer was shown by Zn and (Zn + methotrexate) treatment groups, whereas methotrexate showed the least antibody titer. On day 35, there was a non-significant decrease in the antibody titer ($p=0.195$). Similarly, the antibody titer continued to decrease in all vaccinated groups of rabbits on day 42 and thereafter ($p=0.058$).

The mean lymphocyte count of the rabbits treated with various drugs is shown in Table 2. Different treatment groups showed significant difference in lymphocyte count ($p=0.048$). One week post vaccination, there was a slight increase in lymphocyte count, but various treatments showed statistically non-significant difference ($p=0.403$). On day 14 there was a decline in the lymphocyte count as compared to day 7 but treatments showed significant variation ($p=0.007$). A rise was recorded on day 21 with statistically non-significant difference among the treatments ($p=0.505$).

On day 28 (one week after booster dose), there was a significant rise in lymphocyte count. Means values of different treatments showed significant difference ($p=0.001$). The highest value was shown by Zn + methotrexate and prednisolone treated groups. The methotrexate treated group showed least count as compared with other medicated groups.

In comparison to day 28, a significant decrease in lymphocyte count was recorded from day 35 ($p=0.000$). The same trend was also observed for day 42 and day 49. Both the observations when compared with that of control group showed significant difference ($p=0.001$) and ($p=0.005$), respectively. The lowest count was recorded for methotrexate on day 42 and for prednisolone on day 49. The highest count was shown by (Zn + methotrexate) combination group on day 42.

Table 1: Antibody titer of rabbits that received Newcastle disease vaccine (1ml s/c) at zero day and booster dose on 21 day & 35 day (1ml s/c). The animals received daily doses of various treatments given once daily orally from day 7 to 21 except MTX (dose given in there divided dose, at each administered at 12 h interval)

Treatments	Time (Days)						
	0	7 day	14 day	21 Day	28 Day	35 Day	42 Day
Zn	0	222.8	512	222.8	1024	512	1024
PRD	0	222.9	630.3	512	724.1	362	1024
PRD + Zn	0	181	181	630.3	891.4	315.2	1024
MTX	0	207.9	256	415.9	630.3	512	831.7
MTX + Zn	0	222.9	362	315.2	1024	724.1	1024
Control	0	128	256	512	831.7	831.7	512

Each value presents Geometric Mean Titer (n=6); Zn (Zinc), PRD (prednisolone), MTX (methotrexate)

Table 2: Mean lymphocyte counts of rabbits that received Newcastle disease vaccine (1ml s/c) at zero day and booster dose on 21 day (1ml s/c). The animals received daily doses of various treatments given once daily orally from day 7 to 21 except MTX (dose given in there divided dose, at each administered at 12 hour interval)

Treatments	Time (Days)						
	0	7	14	21	28	35	42
Zn	2494	2632	2182	2405	2573	2033	1405
ZnMTX	2412	2783	2274	2902	3861	2936	2149
MTX	3262	2903	3121	3042	2479	3936	1046
ZnPRD	2709	3124	3030	2520	3333	2296	2049
PRD	2144	2023	2600	3322	4357	3154	1851
Control	2909	3223	1835	2627	2183	2112	1609

Each value presents Geometric Mean Titer (n=6); Zn (Zinc), PRD (prednisolone), MTX (methotrexate)

Table 3: Mean monocytes counts of rabbits that received Newcastle disease vaccine (1ml s/c) at zero day and booster dose on 21 day (1ml s/c). The animals received daily doses of various treatments given once daily orally from day 7 to 21 except MTX (dose given in there divided dose, at each administered at 12 hour interval)

Treatments	Time (Day)						
	0	7	14	21	28	35	42
Zn	112	333	160	141	72	79	51
ZnMTX	85	261	137	130	87	58.5	20
MTX	130	201	162	106	71	122	52
PRD	77	124	102	155	126	13	46
ZnPRD	83	145	185	116	123	72	19
Control	119	333	160	141	72	79	51

Each value presents Geometric Mean Titer (n=6); Zn (Zinc), PRD (prednisolone), MTX (methotrexate)

The mean monocyte count of rabbits of different treatments is shown in table 3. The values at day 0 represent the baseline reading of count of all treatment groups before medication and vaccination. All treatment groups have statistically significant monocytes counts (*p=0.013*). A rise in the count was observed in all treatment groups on day 7 with significant variation (*p=0.000*). There was a decline in the count on the day 14 with no significant variation. The count continued to decrease on day 21 and 28 but difference among the treatment was not significant. On day 35 monocytes showed decline but statically significant variation among different groups (*p=0.011*).

DISCUSSION

Zn and (Zn + methotrexate) treated rabbits showed the highest titer of anti-NDV among all groups. This could be attributed to the multiple effects of zinc on antigen presenting cells, T-cell lymphocytes and the process of antibody genesis (Hasse and Rink, 2009). Methotrexate and prednisolone treated rabbits showed suppression that resulted poor antibody response of the rabbits to NDV antigen. This could be due to reduced synthesis of cytokines, critical in the generation of immune response (Cronstein and Memill, 1996) and (Bernard and Keith, 2001). The higher titer of anti-NDV-HI antibody in all Zn

treated groups at day 35 of study indicated the role of zinc in enhancing the recovery from methotrexate and Prednisolone induced immunosuppression (Hasse and Rink, 2007).

The Higher lymphocytes count in (Zn + prednisolone) and methotrexate medicated rabbits as compared to other groups, indicates that the medicines show predominant effect on physiology of immunocompetant cells rather their count. Zn probably enhances the functions of the lymphocytes or other immunocompetant cells. The results are supported by Fraker *et al.* (2000). On day 42 the decline of lymphocytes in methotrexate treated group reflects the inhibitory action of methotrexate on synthesis of interleukins (Cronstein and Memill, 1996). The lymphocyte count was higher in prednisolone treated rabbits than that of control rabbits but was markedly less than that of Zn + prednisolone treated rabbits (Prasad, 2008). It also indicated that Zn had affected the release of cytokines from monocytes rather their count as evidenced by increased antibody titer (Hajo and Lothar, 2007).

CONCLUSION

Findings of the study suggest that Zn if given concurrently has corrective effect in immunosuppressive and myelosuppressive action of methotrexate,however same cannot be said about the prednisolone.

REFERENCES

Allen WH and Gough RE (1974). A standard hemagglutination inhibition test for newcastlee disease. 1. A comparison of marco amd micro methods. *Vet. Rre.*, **95**: 120-123.

Burgh M Jr. (1978). A simple method for recording and analyzing serological data. *Avain Dis.*, **22**: 362-365.

Chandra RK (1984). Excessive intake of zinc impairs immune response. *JAMA.*, **252**: 1443-1446.

Cook-Mills JM and Fraker PJ (1993). Functional capacity of the residual lymphocytes from zinc-deficient adult mice. *Br. J. Nutr.*, **69**: 835-848.

Corti M, Palmero D and Eiguchi K (2009). Respiratory infections in immunocompromised patients. *Curr. Opin. Pulm. Med*., **15**(3): 209-217.

Cronstein BN, Eberle MA, Gruber HE and Levin RI (1991). Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. *Proc. Natl. Acad. Sci. USA*, **88**: 2441-2445.

Fishman JA (2011). Infections in immunocompromised hosts and organ transplant recipients: Essentials. *Liver Transpl.*, **17**(Suppl 3): S34-S37.

Fraker PJ and King LE (2004). Reprogramming of the immune system during zinc deficiency. *Annu. Rev. Nutr.*, **24**: 277-298.

Frank M Balis, John S Holcenberg, David G Poplack, Jeffrey Ge, Harland N Sather and Robert F Murphy, Matthew M Ames, Mary J Waskerwitz, David G Tubergen, Solomon Zimm, Gerald S Gilchrist and W Archie Bleyer (1998). Pharmacokinetics and pharmaco-dynamics of oral methotrexate and mercaptopurine in children with lower risk acute lymphoblastic leukemia: A Joint Children's Cancer Group and Pediatric Oncology Branch Study. *Blood*, **92**: 3569-3577.

Ghai CL (2007). The total leukocyte count. *In*: Ghai CL,editor. A textbook of practical physiology 7th ed., Jaypee Brother Medical Publishers (Pvt.) Ltd., New Delhi, pp.64-81.

Godeau B, Coutant-Perronne V, Le Thi Huong D, Guillevin L, Magadur G, De Bandt M, Dellion S, Rossert J, Rostoker G and Piette JC (1994). *Pneumocystis carinii* pneumonia in the course of connective tissue disease: Report of 34 cases. *J. Rheumatol*., **21**(2): 246-251.

Hajo H, Dawn JM, Andrew W, Klaus HI, Gabriela E, Silke H, Jonathan RP and Lothar R (2007). Differential gene expression after zinc supplementation and deprivation in human leukocyte subsets. *Mol. Med*., **13**: 362-370.

Hajo H and Lothar R (2007). Signal transduction in monocytes: the role of zinc ions. *Biometals*, **20**:579-85

Ibs KH and Lothar R (2003). Zinc-altered immune function. *J. Nutr*., **133**:1452S–1456S

Lothar R and Hajo H (2007). Zinc homeostasis and immunity. *Trends Immunol*.,**28**:1-4

Neurath MF, Hildner K, Becker C, Schlaak JF, Barbulescu K, Germann T, Schmitt E, Schirmacher P, Haralambous S, Pasparakis M, Meyer Zum Büschenfelde KH, Kollias G and Märker-Hermann E (1999). Methotrexate specifically modulates cytokine production by T cells and macrophages in murine collageninduced arthritis: A mechanism for metho-trexate-mediated immunosuppression. *Clin. Exp. Immunol*., **115**: 42-55.

Philip CC and Samantha K (2002). The immune system: A target for functional foods? *Br. J. Nutr.*, **88**: S165-S176.

Postic B, Catherine D, Mary KB and Monto H (1966). Effect of temperature on the induction of interferons by endotoxin and virus. *J. Bacteriol.* **91**: 1277-1281.

Prasad AS (2008). Zinc in human health: effect of zinc on immune cells. *Mol. Med*., **14**: 353-357.

Prasad AS, Meftah S, Abdallah J, Kaplan J, Brewer GJ, Bach JF and Dardenne M (1988). Serum thymulin in human zinc deficiency. *J. Clin. Invest.*, **82**: 1202-1210.

Tran CD, Howarth GS, Coyle P, Philcox JC, Rofe AM and Butler RN (2003). Dietary supplementation with zinc and a growth factor extract derived from bovine cheese whey improves methotrexate-damaged rat intestine. *Am. J. Clin. Nutr*.,**77**(5): 1296-1303.

Verstappen SM, Bakker MF, Heurkens AH, van der Veen MJ, Kruize AA, Geurts MA, Bijlsma JW, Jacobs JW (2010). Adverse events and factors associated with toxicity in patients with early rheumatoid arthritis

treated with methotrexate tight control therapy: The CAMERA study. *Ann. Rheum. Dis*., **69**(6): 1044-1048.

Weinblatt ME, Coblyn JS, Fox DA, Fraser PA, Holdsworth DE, Glass DN and Trentham DE (1985). Efficacy of low-dose methotrexate in rheumatoid arthritis. *N. Engl. J. Med*., **312**: 818-822.

Wellinghausen N and Lothar R (1998). The significance of zinc for leukocyte biology. *J. Leukocyte Biol.,* **64**: 571-577.

Zhang B and Guo Y (2009). Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. *Br. J. Nutr*., **102**(5): 687-693.

Zitnik RJ, Whiting NL and Elias JA (1994). Glucocorticoid inhibition of interleukin-1-induced interleukin-6 production by human lung fibroblasts: Evidence for transcriptional and post-transcriptional regulatory mechanisms. *Am. J. Respir. Cell Mol. Biol*., **10**: 643-650.

Zurier R and Weissman G (1973). Anti immunologic and anti-inflammtory effects of steroid therapy. *The Med. Clin. North Am.,* **43**: 295.