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EFFECT OF CENTCHROMAN ON CELLULAR AND HUMORAL IMMUNITY

LICTO THOMAS[1], MOHAMMAD ASAD[1]*,
HEREMAGLUR JAGANNATH HRISHIKESHAVAN[1],
GOWDA KALLENAHALLI CHANDRAKALA[2]

[1]*Krupanidhi College of Pharmacy,*
#5, Sarjapur Road, Koramangala,
Bangalore – 560 034

and

[2]*Institute of Animal Health & Veterinary Biologicals,*
Hebbal, Bangalore – 560 024

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Abstract : Centchroman (Ormeloxifene) is a nonsteroidal selective estrogen receptor modulator that is used as once a week oral contraceptive agent. The effect of centchroman on the immune system was evaluated by using different experimental models such as carbon clearance test, cyclophosphamide induced neutropenia, neutrophil adhesion test, effect on serum immunoglobulins, mice lethality test and indirect haemagglutination test. The first three models namely carbon clearance test, cyclophosphamide induced neutropenia and neutrophil adhesion test were used to study cell mediated immunity while the latter three models were used to see the effect on humoral immunity. Centchroman was administered orally at a dose of 5 mg/kg and levamisole (2.5 mg/kg/ p.o) was used as standard drug. Centchroman significantly increased the levels of serum immunoglobulins and also prevented the mortality induced by bovine *Pasteurella multocida* in mice. It also increased significantly the circulating antibody litre in indirect haemagglunation test. However, it did not show any significant effect on phagocytic index in carbon clearance assay and nor did influence the adhesion of neutrophils in the neutrophil adhesion test. Centchroman was also not effective in preventing the cyclophosphamde induced neutropenia. Hence, it was concluded that centchroman increases humoral immunity with no significant effect on cell mediated immunity.

Key words : centchroman humoral immunity cell mediated immunity selective estrogen receptor modulator

INTRODUCTION

Centchroman (Ormeloxifene), is a non-steroidal oral contraceptive and is the only anti implantation agent approved for clinical use in the world. It is chemically 3, 4 trans-7-methoxy-2,2-dimethyl-3-phenyl-4-[4-(2pyrrolidinoethoxy)phenyl]chromanhydrochloride

*Corresponding Author : Dr. Mohammad Asad, Krupanidhi College of Pharmacy, # 5, Sarjapur Road, Koramangala, Bangalore – 560 034; Phone : +91-80-25535751; Fax : +91-80-51309161; E-mail : mohammedasad@rediffmail.com

(1). Apart from its antiimplantation activity, it is reported to have antimutagenic (2), anti-inflammatory (3) and bronchodilator effects (3). It also produces mild respiratory stimulation (3), dose dependent hypotension (3) and stimulates ATPase activity in the liver (3). Centchroman also produced a dose dependent inhibition of osteoclastic bone resorption *in-vitro* when tested at different concentrations (4). It is also known to prevent ovariectomy-induced osteoporosis rats and its use is recommended for the management of post-menopausal osteoporosis in women (5).

Centchroman has been reported to interact with certain drugs. It has been reported that concurrent oral administration of tetracycline (140 mg/kg) twice daily on Days 1–5 postcoitum (pc) interfered with the postcoital antiimplantation activity and almost completely abolished estrogen antagonistic activity resulting in the occurrence of resorbed implantations in 50% of the females (6). Commonly used drugs like ibuprofen, rifampicin, diazepam, salbutamol, nifedipine, paracetamol and haloperidol do not interact with the bioavailability or efficacy of centchroman (7).

Centchroman is effective in the treatment of advanced breast cancer and injectable centchroman is reported to have anti tumor efficacy (8, 9). Centchroman is a selective estrogen receptor modulator. The effect of some of the selective estrogen receptor modulators (SERMs) on the immune system has been studied. Some of the SERMs are known to stimulate the immune system while others are reported to suppress it (10, 11).

The use of centchroman for prevention of conception requires administration for several weeks. A week dose of 30 mg twice for 12 weeks is recommended to build up adequate blood levels or a loading dose of 60 mg should be administered followed by 30 mg doses weekly for minimum 12 weeks (12). Since, centchroman is used for longer periods, the present study was undertaken to evaluate the effect of centchroman on the immune system.

METHODS

Experimental animals

Female albino Wistar rats weighing between 200–250 g and female Swiss albino mice weighing between 25–35 g were used. Institutional Animal Ethics Committee approved the experimental protocol; animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control, and Supervision on Experiments on Animals (CPCSEA). The animals were given pellet food (Lipton India Ltd, Mumbai, India) and water *ad libitum*.

Chemicals

Centchroman (Saheli tablets Batch no. S-02-031) was procured from Hindustan Latex Limited (Ahmedabad, India), levamisole was from Khandelwal Labs (Mumbai, India). Leishmann's stain, Indian ink and gluteraldehyde were purchased from Merck (Mumbai, India). WBC diluting fluid, zinc sulphate and barium chloride were from Nice Chemicals (Cochin, India). Cyclophosphamide (Endoxan Injection) was from German Remedies (Mumbai, India).

Dosage

Centchroman is normally given at a dose of 30 mg twice a day in humans. The animal dose (5 mg/kg b.w) was selected based on the human dose (13).

Treatment

The animals were divided into three groups consisting of six animals each. The first group served as control (vehicle 1 ml/ 100 g p.o), the second group received levamisole (2.5 mg/kg p.o) and the last group was treated with centchroman (5 mg/kg p.d). Mice lethality test had four groups, out of which two served as controls, one positive control and the other negative control. The drug solutions were prepared in distilled water and were administered orally.

Experimental models :

1. *Neutrophil Adhesion test (14,15)*

The rats were treated orally with levamisole and centchroman for 14 days. On day 14, blood samples were collected from the retro-orbital plexus into heparinised vials and analyzed for differential leukocyte count (DLC). After the initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 10 min at 37°C. The incubated blood samples were again analyzed for DLC. The percentage of neutrophils in the treated and untreated blood was determined and the difference was taken as index of neutrophil adhesion.

2. *Mice lethality test (16)*

Female Swiss albino mice were treated with the drug or vehicle orally for 21 days. On the 7th and 17th day of the treatment, the animals were immunized with haemorrhagic septicaemic vaccine (HS vaccine). On the 21st day the animals were challenged subcutaneously with 0.2 ml of lethal dose (25 × LD50) of *Pasteurella multocida* (bovine origin) containing 10^7 cells per ml. The animals were observed for a period of 72 hr and the mortality ratio was determined using the formula.

$$\text{Mortality ratio} : \frac{\text{no. of animals dead}}{\text{total no. of animals}}$$

3. *Cyclophosphamide induced neutropenia (17)*

Female Swiss albino mice received the drug or the vehicle orally for 10 days. On 10th day, neutropenic dose of cyclophosphamide (200 mg/kg *s.c*) was injected and this day was labeled as day zero. Blood was collected, the total leukocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and neutrophil counts (%) in treated groups were compared with the values of the control

4. *Carbon clearance test (18, 19)*

Female albino mice were divided into three groups of six animals each. The animals were treated with the drug or vehicle orally for 5 days. After 48 hr of the last dose of the drug, mice were injected 0.1 ml of Indian ink via the tail vein. Blood samples were withdrawn at 0 and 15 min after injection. A 50 µl blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic

index K was calculated using the following equation :

$$K = (Log_e\ OD1 - Log_e\ OD2)/15$$

Where OD1 and OD2 are the optical densities at 0 and 15 min respectively.

5. *Effect on serum immunoglobulins (20)*

The drugs were administered to female albino rats orally for 21 days. Six hours after the last dose of drug, blood was collected and the serum was used for estimation of immunoglobulin levels using method devised by Mullen.

Briefly, for each serum sample to be analyzed, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution were prepared. To each, 0.1 ml of serum was added from a pipette. They were inverted to enable complete mixing of the reagents and left to stand for 1 hr at room temperature. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulphate ($BaSO_4$) solution. The standard $BaSO_4$ solution was prepared by adding 3 ml of BaCb solution (1.15% w/v) to 97 ml of 0.2N sulphuric acid. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units.

6. *Indirect Haemagglutination Test (IHA test) (14)*

Rats were pretreated with the drugs for 14 days and each rat was immunized with 0.5×10^9 sheep red blood cells (SRBCs) intraperitoneally, including control rats. The day of immunization was referred to as day 0. The drug treatment was continued for another 14 more days and blood samples were collected from each rat at the end of the drug treatment and the titre value was determined by titrating serum dilutions with SRBC (0.025×10^9 cells) in microtitre plates. The plates were incubated at room temperature for 2 hr and examined visually for agglutination. The highest dilution of serum showing haemagglutination was expressed as HA titre.

Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnet's comparison test. The values are expressed as mean ± SEM and $P<0.05$ was considered significant.

RESULTS

1. *Neutrophil Adhesion Test*

Incubation of neutrophils with nylon fibres produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibres. Both centchroman and levamisole did not show any significant increase in the neutrophil adhesion when compared to control (Table I).

2. *Mice Lethality Test*

Administration of *Pasteurella multocida* to control animals produced 100% mortality within 72 hr of administration. Two out of six animals that received vaccination

TABLE I: Effect of centchroman on neutrophil adhesion in rats.

Drug	*Neutrophil (%)*		*Difference*
	Untreated blood	*Treated blood*	
Control	19.16±2.02	12.83±1.13	7.00±1.78
Levamisole (2.5 mg/kg *p.o*)	18.00±2.22	11.16±1.97	6.84±1.01^{ns}
Centchroman (5 mg/kg *p.o*)	19.83±1.77	14.66±1.33	5.17±1.24^{ns}

Values are expressed as Mean±S.E.M, n=6, $^{ns}P>0.05$.

tolerated the lethal dose of the organism and the mortality was 66.66%. Centchroman produced 16.66% decrease in the mortality ratio whereas levamisole showed a 33.33% decrease in the mortality ratio when compared to control.

3. *Cyclophosphamide Induced Neutropenia*

The neutropenic dose of cyclophosphamide reduced the TLC in control animals by 74.75%. Administration of centchroman for 10 days before cyclophosphamide administration produced 44% reduction in TLC while levamisole showed 56% decrease in TLC when compared to initial values. The neutrophil count (%) was reduced by 44% in cyclophosphamide treated control, 17% in centchroman treated animals and 18.65% in levamisole treated animals when compared to initial values. However, statistical analysis revealed that there was no significant difference in the TLC and neutrophil counts before and after cyclophosphamide administration in different groups (Table II).

4. *Carbon clearance test*

Both centchroman and levamisole did not show any significant increase in the phagocytic index when compared to control indicating that there was no increase in the clearance of colloidal carbon from the blood after administration of these drugs (Table III).

5. *Effect on serum immunoglobulins*

Centchroman showed a significant increase in the serum immunoglobulin levels whereas levamisole did not show any significant increase in the serum immunoglobulin levels when compared to control (Table III).

6. *Indirect Haemagglutination test*

The haemmagglutinating antibody (HA) titer value was significantly increased in

TABLE II: Effect on cyclophosphamide induced neutropenia.

Treatment	*Total no. of Leucocytes (cell/mm^3)*		*% reduction*	*% neutrophil*		*% reduction*
	Before	*After*		*Before*	*After*	
Control	5258.3±1108.0	1327.3±209.4	74.75	20.50±2.89	11.50±1.05	44.00
Levamisole	6416.6±1488.0	2825.0±419.8	56.00	18.16±1.22	14.83±1.27	18.65
Centchroman	5366.6±843.2	3041.6±297.0	44.00	22.83±2.52	19.16±1.74	17.00

Values are expressed as Mean±S.E.M, n=6.

TABLE III: Effect on phagocytic index in carbon clearance assay, serum immunoglobulin levels and haemagglutinating antibody titre in IHA test.

Treatment	*Phagocytic index*	*Serum immunoglobulin level (ZST units)*	*HA titer*
Control (1 ml/kg *p.o*)	0.0135±0.0050	21.481±0.3231	26.66±4.21
Levamisole (2.5 mg/kg *p.o*)	0.0101±0.0052	22.191±0.1184	106.66±16.86**
Centchroman (5 mg/kg *p.o*)	0.0290±0.0058	23.826±0.1302**	93.33±13.33**

Values are expressed as Mean±S.E.M, n=6, **P>0.01 when compared to control.

animals that received vaccination along with centchroman or levamisole compared to animals that received vaccination alone (Table III).

DISCUSSION

The results of the present study suggest that centchroman may have action on the humoral immunity as shown by its effect in the indirect haemagglutination test, serum immunoglobulin levels and mice lethality test whereas it may not have much of an effect on the cell mediated immunity as it did not show any significant effect in the neutrophil adhesion test, carbon clearance assay and cyclophosphamide induced neutropenia model.

The adhesion of neutrophils to nylon fibres describes the margination of cells in the blood vessels and the number of neutrophils reaching the site of inflammation (15). Centchroman at a dose of 5 mg/kg, p.o. did not show any significant increase in the neutrophil adhesion to nylon fibres. This indicates centchroman may not increase the migration of neutrophils towards the site of inflammation.

The mouse lethality test is one of several tests used world-wide to evaluate serological responses in animals immunized with vaccines. *Pasteurella multocida* is pathogenic to mice. The mouse lethality test involves injecting mice with the vaccine prior to the administration of the bacterial culture and determining the mortality ratio (21). The vaccination will cause production of antibodies. If the drug has an ability to enhance the production of antibodies to such an extent that antibodies produced can counter the pathogen, then the animal survives. Centchroman showed a 16.66% reduction in the mortality ratio.

The cyclophosphamide induced neutropenia model concentrates on the effect of drugs on the haemopoeitic system (22). Centchroman did not prevent cyclophosphamide induced neutropenia significantly indicating that it may not stimulate the haemopoetic system. The carbon clearance test was done to evaluate the effect of drugs on the reticulo endothelial system. The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells. Cells of the RES are known to be important in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (19). Centchroman and levamisole did not show significant increase in the phagocytic index. Hence, these agents may not have any effect on the reticuloendothelial system.

The estimation of serum immunoglobulin levels is used to evaluate the increase in serum immunoglobulin production after the administration of the drugs. Immunoglobulins are antibodies that react specifically with the antigen. The zinc sulphate turbidity test is used to gain a rough estimation of the amount of immunoglobulins present in the serum. Zinc sulphate causes precipitation of the immunoglobulins making the solution cloudy. A lack of cloudiness signifies lack of immunoglobulins (23). The turbidity is expressed as ZST units which in turn indicate the amount of immunoglobulin present in the sample. Centchroman showed a significant increase in the serum immunoglobulin levels.

The indirect haemagglutination test was performed to confirm the effect of centchroman on the humoral arm of the immune system. The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody secreting cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross linking to form latex that are more readily ingested by phagocytic cells (19). The results showed that the numbers of antibody forming cells, as well as the titers of circulating antibodies are enhanced if the test animals are pretreated with centchroman or levamisole. Centchroman showed highly significant increase in the circulating antibody titre.

The effect of estrogen on the immune system is well known. The female predisposition to autoimmunity and alteration of symptoms of some diseases during pregnancy has led to the idea that lower physiological amounts of estradiol stimulates the immune system whereas pharmacological doses or higher concentration of estradiol inhibits cell mediated immunity (24). As mentioned earlier, the effect of SERMs on some aspects of immune system is documented. SERMs such as tamoxifen, torimifene and raloxifene are known to alter lymphocyte development, immune cell function and cytokine secretion. B-cell lymphopoeisis is inhibited by raloxifene whereas tamoxifen is reported to stimulate mitogen induced immunoglobulin production (10, 11). In the present study, an increase in antibody production was observed as evident from the effect of centchroman on the serum immunoglobulin levels and antibody titre in haemagglutination test. Hence it is suggested that like tamoxifen, centchroman increases antibody production.

To conclude, centchroman stimulates the humoral arm of the immune system in experimental animals. It does not have any significant effect on neutrophil adhesion, macrophage phagocytosis and cyclophosphamide induced neutropenia.

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