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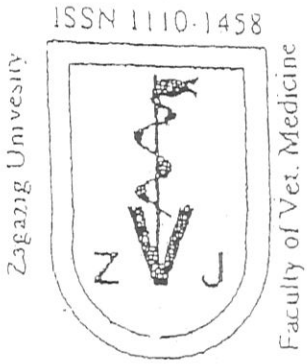

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CONTENTS

The Immunomodulatory Effects of Oral Administration of Azithromycin and *Echinacea spp* in *Pasteurella multocida* Vaccinated Rats

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ABSTRACT

The present study was carried out to investigate the effects of Azithromycin (Zisrocin®) and *Echinacea spp* (Mulone®), alone and their combination on the immune response of the rat as a laboratory model. In this experiment, 40 male rats were employed and divided into four equal groups each of ten. The first group (control group) received 0.5 ml saline. The second group received Mulone® (5 mg/100 gm), the third group received Zisrocin (4.5 mg/100 gm) and the fourth group received Mulone® (5 mg/100 gm) with Zisrocin® (4.5 mg/100 gm) as oral gavage daily for 5 days, respectively. All groups were vaccinated with *Pasteurella multocida* (4×10^9/ml CFU of *P.multocida*) on the 6th day of the experiment then blood samples were collected from all groups at Zero, 1st , 2nd and 3rd day post vaccination. Whole blood and serum samples were collected and used for phagocytic activity, nitric oxide (NO) production and lysozyme activity. The results showed that the combination of Zisrocin and Mulone, synergistically, provoked a significant increase in the phagocytic and lysozyme activities than Zisrocin treated, Mulone treated and control groups, while induced a significant decrease in serum NO level than Zisrocin and control group. Our results concluded that the simultaneous use of Zisrocin and Mulone improved the immune and the anti-inflammatory responses in vaccinated rats.

INTRODUCTION

Macrolides are broad spectrum antibacterial, widely used in the treatment of respiratory tract infections *(1)*. Azithromycin (one of macrolides) shows anti-inflammatory actions in several animal models had acute inflammation and clinical benefit when given for several months in diffuse panbronchiolitis, asthma and cystic fibrosis in human *(2,3)*.

Echinacea purpurea (EP) has become one of the popular herbal products in North America and Europe as an immune promoter, particularly for prevention and treatment of upper respiratory tract infections *(4,5)*.

In the present study, the effects of Zisrocin and Mulone administration on the immune and anti-inflammatory responses of the albino rat were compared, alone and in combination.

MATERIAL AND METHODS

Drugs

Azithromycin: (Zisrocin®)

Zisrocin® suspension was developed by EGYPHAR, Obour City- Egypt. Azithromycin powder for oral suspension, when reconstituted, each 5 ml contains Azithromycin dehydrate equivalent to 100 mg azithromycin base. The recommended dose is 4.5 mg/ 100 gm *(6)*.

B- *Echinacea spp*: (Mulone®):

Mulone® drops produced by ATOS PHARMA, Cairo-Egypt. Each 30 ml contain: dry extract of Echinacea 1.125 gm. The recommended dose is 5 mg/100 gm *(7)*.

Animals

Fourty male Albino rats weighing 180–200 g were obtained from laboratory Animal House, Faculty of Veterinary Medicine,

Zagazig University. They were fed milk and barely and acclimatized for 2 weeks before starting the experimental study. After that, the animals were randomly divided into four groups: Control, Zisrocin®, Mulone® and Zisrocin + Mulone of 10 rats each. The first received 0.5 ml saline and kept as a control, the second received Zisrocin® (4.5 mg/100gm) *(6)*, the third received the Mulone® (5 mg/100 gm) *(7)*, and the fourth received the combination of Zisrocin® (4.5 mg/100gm) and Mulone® (5 mg/100 gm). All drugs were administered to the animals by oral gavage for 5 days.

Vaccine

All rats were vaccinated with formalized killed *Pasteurella multocida* (4×10^9/ml CFU of *P.multocida*) obtained from **Vaccine and Serum Institute (Abbasia)** one day after the end of drug administration.

Blood sampling

Immune functions were assessed in animals on zero, 1st, 2nd and 3rd day post vaccination using blood and serum samples obtained from the slaughtered animals. From each animal, one blood sample was collected with EDTA as anticoagulant on the 1st day post vaccination for phagocytic assay *(8)*. Second sample was collected without anticoagulant for serum separation on Zero, 1st, 2nd and 3rd day post vaccination for NO production *(9)*.and Lysozyme activity test *(10)*.

Statistical analyses

Data were statistically analysed using the computer program (SPSS version 15 for Windows), and comparisons were made using one and two ways ANOVA. If significant differences between means were found, Duncan's multiple range test, whose significant level was defined as ($P \leq 0.05$), was used *(11)* to estimate the effect of different treated groups.

RESULTS

Phagocytic Assay

It was clearly evident from Table 1 that the oral administration of Zisrocin ® (4.5 mg/100gm), Mulone ® (5 mg/100 gm) and their combination in their recommended doses for 5 days elicited a significant ($P \leq 0.05$) increase in the mean values of phagocytosis percent and index when compared with vaccinated group. The rank order of the increase was as follows: The combination of the 2 drugs followed by Mulone and then Zisrocin.

Table 1. Effect of oral administration of Zisrocin; 4.5mg/100gm, Mulone; 5mg/100gm and their combination in the same dose for 5 days (Mean±SE) on phagocytosis % and index in *Pasteurella multocida* vaccinated rats (N=5).

Group	Vaccinated	Zisrocin	Mulone	ZIS+Mul
Phagocytosis %	43^{d}±2.20	60^{c}±1.50	79^{b}±.73	93^{a}±2.45
Phagocytosis index	2.26^{d}±0.19	4.12^{c} ±0.30	6.62^{b}±0.23	8.54^{a}±0.32

Means carrying different litters are significant at $P \leq 0.05$.

Lysozyme activity Assay

Table 2 illustrates that vaccination with formalized killed *Pasteurella multocida* vaccine afforded a significant ($P \leq 0.05$) elevation after 1st , 2nd day post vaccination, whereas, the lysozyme activity returned back to nearly the value at Zero day meanwhile all the treated group showed a significant ($P \leq 0.05$) increase in serum lysozyme activity on Zero, 1st, 2nd and 3rd days post vaccination compared with vaccinated group except with Zisrocin on the 2nd and 3rd days post vaccination which revealed a slight increase with the rank order of potency as follows: the combination of the 2 drugs followed by Mulone and lastly Zisrocin.

Table 2. Effect of oral administration of Zisrocin; 4.5mg/100gm, Mulone; 5mg/100gm and their combination in the same dose for 5 days (Mean±SE) on serum lysozyme activity in *Pasteurella multocida* vaccinated rats (N=5).

DPV	Vaccinated	Zisrocin	Mulone	ZIS+Mul
Zero	142.67 ^{f}±5.40	177.98 ^{de}±6.98	195.63 ^{cd}±8.25	204.45 ^{bc}±16.21
1st	177.98 ^{de}±6.98	204.45 ^{bc}±8.25	226.51 ^{ab}±12.86	239.75^{a}±8.26
2nd	177.98 ^{de}±6.98	191.21 ^{cd}±11.25	208.86 ^{bc}±5.40	213.28 ^{bc}±8.83
3rd	147.09 ^{f}±5.40	160.33 ^{ef}±8.26	173.56 ^{de}±8.26	177.98 ^{de}±9.87

Means carrying different litters are significant at ($P \leq 0.05$).
DPV: Days Post Vaccination

Nitric Oxide Assay

Table 3 showed that the administration of Zisrocin, Mulone and their combination increased significantly ($P \leq 0.05$) the nitric oxide production on Zero time compared with vaccinated group. Meanwhile on the 1st and 2nd day post vaccination Zisrocin induced a significant ($P \leq 0.05$) increase in the NO production compared with vaccinated group, yet Mulone and its combination with Zisrocin elicited a significant ($P \leq 0.05$) decrease on the 1st and 2nd day post vaccination whereas, non-significant changes were recorded after 3rd day post vaccination. These results indicate that Mulone antagonize NO release by Zisrocin, thus the concurrent use of the 2 drugs have anti-inflammatory effect and decrease host tissue damage induced by NO release.

Table 3. Effect of oral administration of Zisrocin; 4.5mg/100gm, Mulone; 5mg/100gm and their combination in the same dose on (Mean±SE) serum NO production in *Pasteurella multocida* vaccinated rats (N=5).

DPV	Vaccinated	Zisrocin	Mulone	ZIS+Mul
Zero	5.84 ^{j}±0.26	14.44 ^{cd}±0.25	10.63 ^{fg}±0.41	13.13 ^{cde}±0.41
1st	17.13 ^{b}±0.34	26.64 ^{a}±2.70	12.16 ^{ef}±0.22	12.56 ^{def}±0.37
2nd	13.16 ^{cde}±0.11	14.99 ^{bc}±0.77	8.04 ^{hi}±0.31	8.86 ^{gh}±0.28
3rd	7.59 ^{hig}±0.33	8.17 ^{hi}±0.48	6.63 ^{ij}±0.37	6.65 ^{ij}±0.55

Means carrying different litters are significant at ($P \leq 0.05$).
DPV: Days Post Vaccination.

DISCUSSION

Echinacea purpurea is best known for its immunomodulatory effects *(12, 13)*. Macrolide is the antibiotic class with more convincing studies and evidence on its immunomodulatory and anti-inflammatory activities *(14)*.

In the present investigation, it has been observed that vaccination of rats with formalin killed *Pasteurella multocida* vaccine induced a significant increase in serum nitric oxide (NO) and lysozyme levels on the 1st and 2nd day post vaccination assuring the efficacy of this vaccine in rats.

The present data were in accordance with that recorded by **Hussainin** *(15)* who reported that pooled serum sample from rats vaccinated with live and killed form of the clone was administered to mice, provided 66% protection when compared with active immunization with

the recombinant clone which conferred 83% immunity to mice when challenged with lethal dose of P. multocida. ELISA results were positive for presence of antibody in serum of immunized mice.

The results of the present work reported that oral administration of Echinacea provoked a significant increase in the phagocytic activity including phagocytosis percent and phagocytic index.

A series of studies in mice using purified *Echinacea purpurea* (EP) plant showed a stimulatory effect when applied to immune cells in culture or injected intraperitoneally into mice. These effects showed an increase in phagocytosis, chemotaxis, and oxidative burst of either neutrophils or macrophages *(5, 16, 17)*.

The present study data is clearly reinforced with those previously obtained by **Khaksary** *(18)* who found, in one double-blind study, that oral administration of EP extracts increased polymorphonuclear phagocytic activity for 5 days. On the same line, it has been reported reported that phagocytosis in mice was enhanced after treatment with ethanolic extracts of EP *(19)*.

In the same line, the phagocyte activity of EP in the test groups was significantly higher than that of the control group during all study days *(20)*.

Apparently, purified polysaccharides, cichoric acid and alkylamides from EP act on the non-specific branch of immunity, the phagocytic cells, rather than the specifically acquired branch *(4, 5)*.

In the current study, it has been shown that oral administration of Azithromycin provoked a significant increase in the phagocytic activity including phagocytosis percent and phagocytic index.

Similar outcomes have been observed *(3)* which reported that low-dose azithromycin improves the ability of both alveolar (AM) and monocyte-derived macrophages (MDM) to phagocytose bacteria. This further supports the long-term use of low dose azithromycin as an attractive adjunct treatment option for reducing inflammation, bacterial colonization and exacerbation rate in COPD.

The results obtained in the present study revealed that oral administration of Echinacea for 5 days elicited a significant increase in serum lysozyme level on the Zero, 1st and 2nd days post vaccination.

In human lung, lysozyme is secreted by serous cells of the submucosal glands and by airway epithelial cells *(21, 22)*. Immunodepletion of lysozyme has been proved to decrease antibacterial activity in human airway and nasal secretions by about 50%, providing *in vivo* evidence that lysozyme is a major component of airway host defense *(23)*.Lysozyme plays an important role in innate host defense of the lungs *(24)*.

Our results go hand in hand with those which showed that *(25)* who found that Echinacea alkamide (Dodeca-2,4,8,10-tetraenoic acid isobutylamide) was able to enhance significantly the release of lysozyme activity in THP-1 monocytes with an increase of up to 92% after one hour exposure. The data obtained suggested the significant stimulatory effect of Echinacea alkamide on lysozyme secretion of monocytes as a novel mechanism of action for the immunomodulatory properties of this bioavailable phytochemical of Echinacea.

In our work, oral administration of Azithromycin for 5 days elicited a significant increase in serum lysozyme level on the Zero, 1st and 2nd day of vaccination. This data was in agreement with **Abdelghaffar** *(26)* who noted that two 14-membered-ring macrolides, roxithromycin and clarithromycin and the azalide azithromycin, but not various 16-membered-ring macrolides or oleandomycin, promoted neutrophil exocytosis of lysozyme and in a time and concentration dependent manner. In a preliminary study performed before *(27)*, they observed a similar effect with erythromycin, erythromycylamine and dirithromycin, three14-membered-ring macrolides.

The mechanism underlying the degranulating effect of macrolides remains to be elucidated. Intracellular accumulation appears to be necessary, as suggested by the observation that experimental conditions which favour macrolide uptake also favour the degranulating effect. In particular, alkalinization of the medium significantly enhances the exocytosis stimulated by erythromycylamine and dirithromycin *(26),* A similar effect was shown with azithromycin.

Similar effects were previously observed *(28)* which indicated that a 3-day treatment of healthy human subjects, with a standard anti-bacterial dosage regimen of azithromycin, exerts acute effects on the release of neutrophil granular enzymes.

In the present study, oral administration of Echinacea for 5 days induced a significant increase in serum Nitric Oxide (NO) level on the Zero day of vaccination while induced a significant decrease in NO level on the 1st and 2nd day post vaccination.

NO and TNF-are two key mediators in host defense and inflammatory response. They positively regulate each other *(29, 30)* and therefore amplify inflammatory signals. However, improper upregulation of these inflammatory players are implicated in a pathological role in inflammatory processes *(29, 31)*.

The results of Zero day of vaccination are similar to that observed by **Rininger** *(32)*, who reported that, In non-activated macrophage, *Echinacea purpurea* induced secretion of macrophage-derived cytokines: IL-1a, IL-1b, IL-6, and IL-10, as well as NO production in a time- and dose-dependent fashion. This would potentiate innate immune response.

On the other hand, the data of the 1st and 2nd day post vaccination were in accordance with that reported before *(33-35)* which demonstrated the suppressive effects of Echinacea extracts and certain individual fractions on production of NO and TNF in an activated macrophage cell line.

Echinacea pallida (EPA) at 200 µg/ml simultaneously increased the potential of bacterial killing but inhibited NO production by macrophages *(36)*. A reasonable explanation is that macrophages may depend on multiple mediators (i.e. reactive oxygen intermediates), not just NO, to provide them with *Salmonella*-killing activity *(37)*.

An alcohol tincture from E. purpurea roots increased the nuclear expression of multiple pro-inflammatory transcription factors (e.g. NF-κB and STATs) in non-activated human bronchial epithelial cell line BEAS-2B, but inhibited the expression of these transcription factors when the cells were infected with rhinovirus *(38)*, thus providing strong mechanistic evidence to explain the observed phenomena in our study and in **Zhai** *(35)*study; that is, Echinacea extracts have different influences on the non-activated and activated macrophages, and the effects of Echinacea on the inflammatory mediators are associated with the modulation of transcription factor expression.

In stimulated macrophages, certain individual alkamides could inhibit NF-kB activity. Down-regulation of NF-kB and inducible Nitric Oxide Synthase iNOS expression will lead to decrease NO production *(34)*.

In our work, the stimulatory effect of orally administered Mulone on Candida phagocytosis may indicate that Echinacea will not adversely affect the important innate immune functions even after inhibition of NO production. This indicates that Echinacea has a beneficial anti-inflammatory effect.

Our data showed that, Azithromycin and Mulone had a synergistic stimulatory effect on innate immunity by increasing phagocytosis percent Table (1), index Table (2) and serum lysozyme activity Table (3) and these effects were more noticeable than in other groups. In addition, the combination of the 2 drugs decreased the serum NO level than other groups suggesting a potent anti-inflammatory effect.

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الملخص العربى

التأثير المناعي للأزيسروميسين والاكينيسيا في الجرذان المحصنة بلقاح الباستيريلا مالتوسيدا

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تم عمل هذه الدراسة لبيان تأثير كل من الازيسروميسين (الزيسروسين), الاكينيسيا (المولون) وخليط منهما علي الاستجابة المناعية في الجرذان المحصنة بلقاح الباستيريلا مالتوسيدا. تم استخدام ٤٠ من ذكور الجرذان البالغة, قسمت الي ٤ مجموعات متساوية كل مجموعة ١٠ جرذان. المجموعة الاولي (المجموعة الضابطة) تم تجريعها ب ٠,٥ ملي محلول ملحي. أما المجموعات الثلاث الاخري فجرعت بالمولون (٥ مجم/ ١٠٠ جم), الزيسروسين (٤,٥ مجم/ ١٠٠ جم) وخليط منهما بنفس الجرعات السابقة لمدة ٥ ايام , علي الترتيب. تم تحصين كل المجموعات بالباستيريلا مالتوسيدا في اليوم التالي لانتهاء التجريع. تم تجميع عينتان من دم كل جرذ الاولي بمانع للتجلط لقياس قدرة الخلايا المناعية علي الالتهام والعينة الثانية بدون اضافة مانع للتجلط لجمع المصل لدراسة التأثير علي نشاط الليزوزيم وانتاج اوكسيد النيتريك.

أظهرت النتائج ان خليط الزيسروسين والمولون ادي الي ذيادة معنوية في القدرة علي الالتهام ونشاط الليزوزيم بالمقارنة بالمجموعة الضابطة وكل دواء علي حدي. بينما ادت الي نقص معنوي في في انتاج اوكسيد النيتريك بالمقارنة بمجموعة الزيسروسين والمجموعة الضابطة. ويستخلص من هذه الدراسة ان خليط الزيسروسين والمولون يحسن الاستجابة المناعية والتأثير المضاد للالتهاب في الجرذان المحصنة عن كل دواء علي حدي.