G.Ü. Fen Bilimleri Dergisi, 16(2):233-238, 2003 ISSN 1303-9709 *G.U. Journal of Science 16(2):233-238, 2003*

THE EFFECTS OF 20-HYDROXYECDYSONE ON HEMOCYTES OF *GALLERIA MELLONELLA* (LEPIDOPTERA) *IN VITRO* CONDITIONS

Savaş İZZETOĞLU, Sabire KARAÇALI*

Ege Üniversitesi, Fen Fakültesi, Biyoloji Bölümü, Moleküler Biyoloji Anabilim Dalı, 35100, Bornova-İzmir, TÜRKİYE, karacali@sci.ege.edu.tr

ABSTRACT

The effects of different concentrations of 20-hydroxyecdysone (20-E) on hemocytes of *Galleria mellonella* were investigated *in vitro*. Hemocyte culture was performed in Grace's insect medium with fetal calf serum. The hormone was added to the cultures at two different concentrations. During a period of three days, the cells (plasmatocytes, granulocytes and total cell number) were counted under an inverted microscope. Obtained numbers were evaluated with one way variance analysis (ANOVA). Hormone treatment led to a dose-dependent decrease in total cell and granulocyte number. The number of plasmatocytes, which are the predominant cells for phagocytose activity in immune response, was enhanced by hormone treatment.

Key Words: 20-hydroxyecdysone, hemocyte, in vitro, Galleria mellonella, insect

20-HİDROKSİEKTİZONUN *IN VITRO* KOŞULLARDA *GALLERIA MELLONELLA*'NIN (LEPİDOPTERA) HEMOSİTLERİNE ETKİLERİ

ÖZET

20-hidroksiektizonun (20-E) *Galleria mellonella*'nın hemositlerine etkileri *in vitro* da araştırıldı. Hemosit kültürü fetal calf serumlu Grace'in böcek kültür ortamında gerçekleştirildi. Hormon kültürlere iki farklı konsantrasyonda verildi. Üç gün boyunca, hücreler (plazmatositler, granulositler ve toplam hücre sayısı) inverted mikroskop altında sayıldı. Elde edilen sayılar tek yönlü varyans analizi ile (ANOVA) değerlendirildi. Hormon uygulaması toplam hücre ve granulosit sayısında doza bağlı bir azalmaya neden olmuştur. İmmün yanıtta fagositoz aktivitesi bakımından en etkili hücre olan plazmatositlerin sayısı hormon uygulaması ile artmıştır.

Anahtar Kelimeler: 20-hidroksiektizon, hemosit, in vitro, Galleria mellonella, böcek.

1.INTRODUCTION

Insects have an active immune system in order to survive in habitats that are highly infected with microorganisms. Research on insect immunity has got enormous interest due to the fact that insects are the most important for humans as vectors of many diseases. Knowledge of insect

defense system can provide new strategies for the biological control of the insect pests or vectors (1).

Insect immune systems consist of molecules, cells and mechanisms. The immune defense against the potential pathogens is composed of humoral and cellular mechanisms (1-4). The humoral immune response is characterized by rapid and transient synthesis of immune proteins. The humoral factors react with foreign surfaces to damage or mark them for cellular attack (1,3). The cellular immune response reacts against pathogens, parasites or eggs by phagocytosis (invading organisms by single hemocytes), multicellular encapsulation and nodule formation (1,3,4,5,6,7). The cellular defense system is based on hemocytes circulating in hemocoel. The system is able to distinguish between self and non-self. (1,7,8). Granulocytes and plasmatocytes are important in recognition of pathogens and parasites. They are the effector cells to the responses of these defense reactions. Plasmatocytes are the predominant cells for phagocytose behavior. Granulocytes possess a small phogocytic activity (3,8,9).

Insect tissue culture has a long history beginning in 1915 (10). Although this began early, culture was carried out by a few scientists. In contrary to mammalian cells, insect cells usually do not grow in a continuous culture. For this purpose, various media have been developed. They are suitable for short-term maintenance of hemocytes. Production of hemocyte lines for continuous *in vitro* has not been successful because mitosis occurs rarely (3,10). Addition of insect body fluids or tissue extracts and own hormones to the media have been helpful for establishing the insect cell lines. Especially, insect molting hormone ecdysone and 20-hydroxyecdysone (20-E) were the most influence to the mitotic rate. Ecdysone releasing from prothoracic glands controls the molting processes during post embryonic developments. 20-E is used as a growth-promoting factor in cell culture and causes to more alterations on cell lines such as; proliferation, morphogenesis, biochemical changes, and increases in phagocytosis and lysis (11-16).

In this work, the effects of 20-E on hemocytes which play an important role in immune response were investigated *in vitro* conditions. The other aim of the study was to determine hemocyte culture conditions required for future studies on insect immunity.

2. MATERIAL and METHOD

The great wax mouth, *Galleria mellonella* (Lepidoptera; Pyrallidae) was used in this study. Larvae were reared on an artificial diet (18-20) at 30±2 °C in darkness, natural relative humidity within large size petri dishes. Hemocytes (insect blood cells) obtained from last instar larvae were used for the experiments.

Cell culture medium was composed of Grace's insect medium supplemented with fetal calf serum (Sigma, f-3018) and antibiotic-gentamycin (GIBCO). 2 ml cell culture medium and 1 ml medium with hemolymph were put to each flask. 20-hydroxyecdysone (20-E) (Rohto-Pharmaceutical) was added to the selected cultures at concentrations of 10 µL (16.7 µg/mL active matter) and 30 µL (50 µg/mL active matter). The cells were maintained at 26 °C and natural relative humidity in 25 cm^2 tissue culture flasks.

The experiments were repeated twice. Hemocytes were counted at randomly determined regions that were the same all flasks under the inverted microscope in the course of three days. Cells in question were first counted approximately after 30 min-incubated.

The experimental data was evaluated with statistical programs, so-called STATGRAF and STATISTICA 5.0 on a computer and also analyzed for the statistical significance. Obtained numbers for the 3-days were tested by one way analysis of variance (ANOVA). The Student's t-test was applied to among the combined data obtained from the repeated groups (combined criteria $p \leq 0.05$) (Table 1).

Table 1: Results of student's t-test (P: Statistically significant P<0.05, t0.05(9): 2.262)

Experiments		*Plasmatocyte*			*Granulocyte*			*Total Hemocyte*		
Control I-II	P	0.497	0.299	0.905	0.296	0.450	0.542	0.446	0.168	0.600
	t.cal	0.693	1.068	0.121	1.077	0.772	0.622	0.778	1.436	0.534
10µL 20E I-II	P	0.282	0.175	0.154	0.234	0.799	0.747	0.419	0.409	0.351
	t.cal	1.109	1.411	1.489	1.233	0.258	0.327	0.826	0.845	0.956
30µL 20E I-II	P	0.101	0.115	0.080	0.558	0.780	0.700	0.131	0.222	0.361
	t.cal	1.730	1.655	1.857	0.597	0.284	0.391	1.580	1.265	0.936
	Days	*1.*	*2.*	*3.*	*1.*	*2.*	*3.*	*1.*	*2.*	*3.*

3. RESULTS

Hemocytes obtained from the last instar larvae of *G.mellonella* were used in all experiments. Effects of 20-hyroxyecdysone (20-E) were studied *in vitro* conditions. In order to obtain the number of plasmatocytes, granulocytes (Fig. 1a,b) and total cell, all cell types were counted respectively.

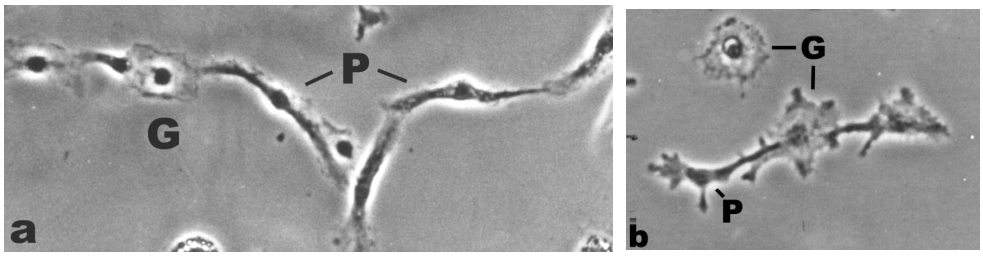

Figure 1. Hemocytes in culture. P: plasmatocyte, G: granulocyte. (a: x161.7, b: x173.2)

According to our counting, total cell number of control group was increased within the second days. The increase was continued in the third days. Treatment of low dose 20-E led to a statistically important increase in total cell number in the second days. In the third days, the increase was continued but the value was not statistically meaningful. After treatment of higher dose of 20-E, obtained results in total cell number were similar to the ones found in low dose treatment for the second and third days. Interestingly, according to comparison of results in third days, hormone treatments resulted in reduction of cell numbers at concentrations 10 µL and 30 µL as compared with control groups (Fig. 2A).

When cell types were compared separately, different effects of 20-E were observed on granulocytes and plasmatocytes. In the control cultures, a much stronger enhancement was found in the number of granulocytes. In the second and third days cell number was significantly increased. After day, there were no significant increases. In both hormone treatments, the number of granulocyte was increased statistically in the second days but no significant change was found in the following days. Although these increases appear important in second days, the values were smaller than that of their control values. As a result of hormone applications, 20-E caused cell numbers to reduce. The negative effect was more conspicuous when hormone concentration was enhanced (Fig. 2B).

According to our counting, proliferation rate of plasmatocytes was lower than granulocytes (Fig. 2B,C). Applications of 10 µL and 30 µL 20-E increased dramatically

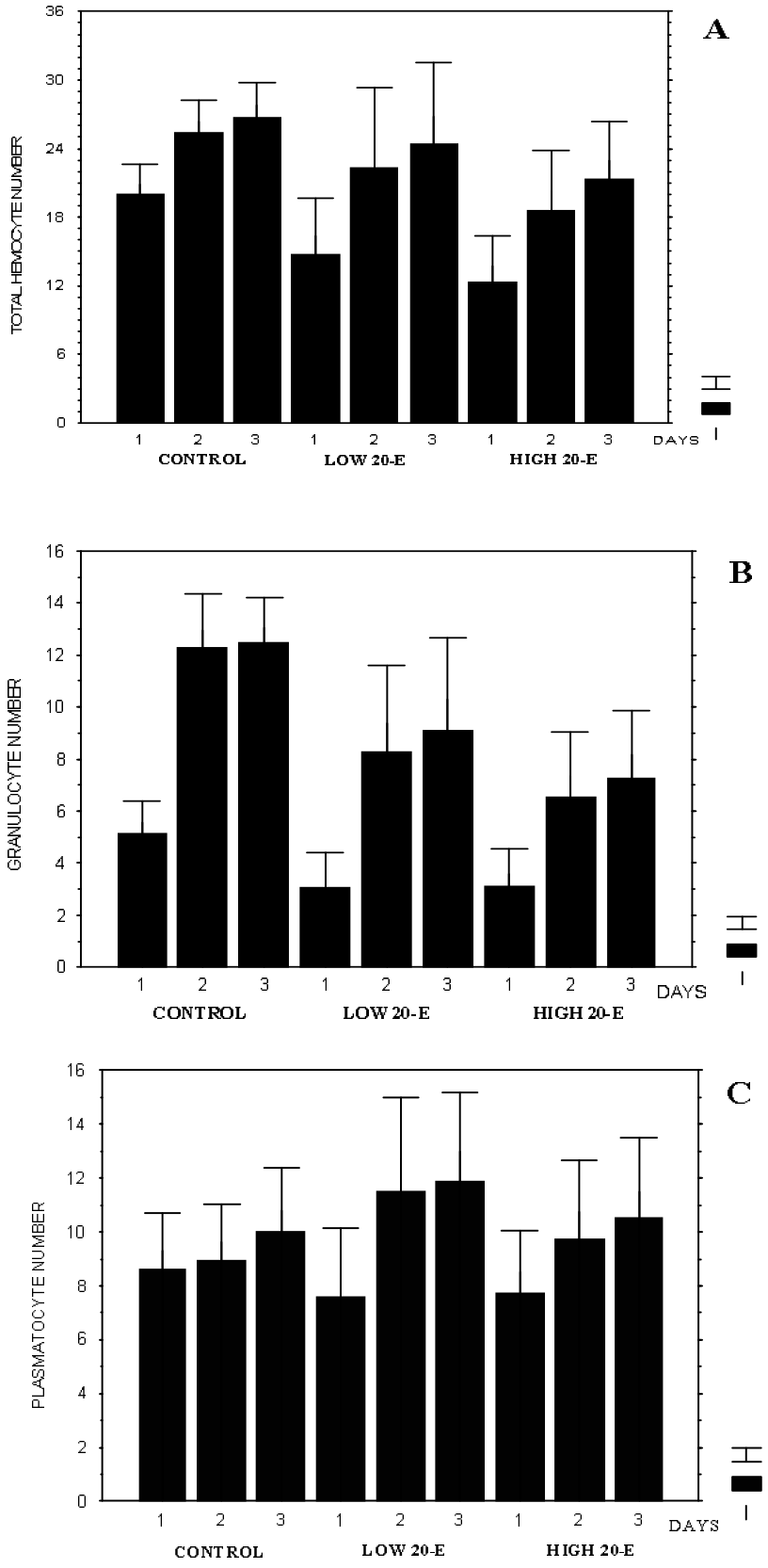

Figure 2. Comparison of the effects of 20-hydroxyecdysone (20-E) on hemocytes number at low and high concentrations (A total hemocyte, B granulocyte and C plasmatocyte)

plasmatocyte number in the second days when compared to the control group. Interestingly, at low dose of 20-E treatment was more effected on these cells. In comparison of results during three days, hormone treatments promoted plasmatocyte number especially at the concentration of low dose 20-E (Fig.2C).

As a result, hormon treatments reduced the rate of total cell number. Essentially, similar results were obtained on granulocyte number in presence of the same hormone concentrations. These findings showed that hormone treatment cause the dose-dependent to decrease in total cell and granulocyte number. In contrast, hormone application enhanced the number of plasmatocytes. This effect was inhibited in low dose of 20-E. However, plasmatocyte number was not able to prevent reducing total cell number.

4.DISCUSSION

Most of the tissue culture studies of insect hormones have focused on ecdysteroids (20). Various forms of ecdysone, especially 20-hydroxyecdysone (20-E) have been added to insect cell cultures as a growth-promoting activity (14). First report of the effects of ecdysone on cultured lepidopteran imaginal disc cells in vitro was carried out by Oberlander and Fulco (1967). Recently, studies in the field of insect immunology and endocrinology were led to development of cell culture methods for the research of hormonal action and immune system (20).

Different amounts of the hormone were applied to various insect cultures. According to the treatments for studying the effects of 20-E, generally there were significantly different results obtained on proliferation among the ecdysone doses. For example, midgut stem cells of *Manduca sexta* and wing disc cells of *Bombyx mori* were stimulated and entered mitosis by the presence of 20-E *in vitro* concentrations of 0.001 µg/mL and 0.01 µg/mL respectively (21.22). In contrast, when the concentration was increased, a reduction of cell division depending on the concentration of 20-E was obtained in some insects. Wing disc cells of *B. mori* were suppressed at 10-fold higher concentration than 0,01 µg/mL (22). Furthermore, IPLB-Tcon 1 cells of *Tichogramma confusum* had slower growth rates when ecdysone concentration increased from 0,01 µg/mL to 10 µg/mL (12). Similar result was obtained from hemocytes of *Galleria mellonella* using two different concentration of 20-E. As reported in this study, hormone treatments had reduced of the cell number particularly in total cells and granulocytes as shown in Figure 2. The result was strongly confirmed by the effects of high dose 20-E *in vitro*.

Another effect of 20-E in cell culture conditions is to enhance phagocytose activity. Plasmatocytes and granulocytes are the most efficient of hemocytes in cellular defense mechanisms of insects (1,3,7,9,23,24). In accordance with studies related to the subject, ecdysone treatments in vitro increased phagocytic capacity of l(2)mbn cells and mutant blood cells of *Drosophila melanogaster* (16, 25). Plasmatocytes are the predominant cells for phagocytose behavior (3,9) and their number of *G. mellonella* was enhanced by hormone treatment in the present study.

5. ACKNOWLEDGEMENTS

The authors want to thank E.U. Research Fund (98 Fen 040) for their partial financial support.

REFERENCES

1. Vilkinskas, A. and Götz P., "Parasitic fungi and their interactions with the insect immune system", ***Adv. Parasitol.***, 43 (1999).
2. Wiesner, A., Losen, S., Kopacek, P., Weise, C. and Götz, P., "Isolated apolipophorin III from *Galleria mellonella* stimulates the immune reactions of this insect", ***J. Insect Physiol.*** 43(4): 383-391 (1997).
3. Lackie, A.M., "Haemocyte behaviour", ***Adv.Insect Physiol***., 21 (1988).
4. Ratcliffe, N.A., Rowley, A.F., Fitzgerald, S.W. and Rhodes C.P., "Invertebrate immunity: basic concepts and recent advances", ***Int. Rev. Cytol***., 97 (1985).

5. Jarosz, C., "Active resistance of entomophagous rhabditid *Heterorhabditis bacteriophora* to insect Immunity", ***Parasitol.***, 117:201-208 (1998).

6. Trenczek, T. and Kanost, M.R., "Insect haemocytes-multifunctional cells in defence mechanisms" ***Sbornik Jihoceske Univerzity Zemedelske Fakulty V Ceskych Budejovicich***, Fytotechnica 2(XIV):18 (1997).

7. Yokoo, S., Götz, P. and Tojo, S., "Phagocytic activities of haemocytes separated by two simple methods from larvae of two lepidopteran species, *Agrotis segetum* and *Galleria mellonella*", ***Appl. Entomol. Zool.,*** 30(2): 343-350 (1995).

8. Karaçalı, S., Deveci, R., Pehlivan, S., and Özcan, A. "Adhesion of hemocytes to desialylated prothoracic glands of *Galleria mellonella* (Lepidoptera) in the larval stage" ***Invert. Reprod. Dev.***, 37:2, 167-170, (2000)

9. Pech, L.L. and Strand, M.R., "Granular cells are requıred for encapsulation of foreign targets by insect haemocytes", ***J. Cell Sci.***, 109: 2053-2060 (1996).

10. Oberlander, H. and Ferkovich, S.M., "Physiological and developmental capacities of insect cell lines" insect cell biotechnology" ***Edited by Maramorosch***, K., McIntosh, A.H., 7:129-140 (1994).

11. Oberlander, H., Silhacek, D.L. and Porcheron, P., "Non-steroidal ecdysteroid agonists: tools for the study of hormonal action", ***Arch. Insect Biochem. Physiol.***, 28:209-223 (1995).

12. Lynn, D.E. and Hung Akey, C.F., "Development of continuous cell lines from the egg parasitoids *Trichogramma confusum* and *T. exiguum*" ***Arch. Insect Biochem. Physiol***., 18:99-104 (1991).

13. Lynn, D.E., Oberlander, H. and Porcheron, P., "Tissues and cells in culture" ***Microscopic Anatomy of Invertebrates***, 43(11): 1119-1141 (1998).

14. Bayne, C.N., "Invertebrate cell culture considerations: insects, ticks, shellfish, and worms", ***Methods in Cell Biology***, 57 (1998).

15. Lynn, D.E., Feldlaufer, M.F., and Lusby, W.R., "Isolation and identification of 20-hydroxyecdysone from a lepidopteran continuous cell line", ***Arch. Insect Biochem. Physiol.***, 5:71-79 (1987).

16. Dinan, L., "Ecdysteroid recptors in a tumorous blood cell line of *Drosophila melanogaster*", ***Arch. Insect Biochem. Physiol.***, 2:295-317 (1985).

17. Marston, N., Campbell, B. and Boldt, P.E., "Mass producing eggs of the greater wax moth, *Galleria mellonella* (L.)", ***Technical Bulletin***, 1510, U.S.Dept. of Agriculture, 1-15 (1975).

18. Ergezen, S. and Özden, Y., "The study of hormonal regulation on the differential protein synthetic activities of fat body in last larval stage of *Galleria mellonella*", ***Türkiye Bilimsel ve Teknik Araştırma Kurumu Temel Bilimler Araştırma Grubu***, Proje No: TBAG-589 (1985).

19. Akçelik, M., "Büyük mum güvesi (*Galleria mellonella* L.) yumurtalarının açılması ve larval gelişme", T***eknik Arıcılık***, 9:25 (1987).

20. Oberlander, H., Leach, C.E., and Shaaya, E. "Juvenile hormone and juvenile hormone mimics inhibit proliferation in a lepidopteran imaginal disc cell line", ***J. Insect Physiol.***, 46:259-265 (2000).

21. Sadrud-Din, S., Loeb, M.J. and Hakim, R.S., "In vitro differentiation of isolated stem cells from the midgut of *Manduca sexta* larvae", ***J. Exp. Bio.,*** 199: 319-325 (1996).

22. Sakurai, S., Kaya, M. and Satake, S., "Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval-pupal metamorphosis and a reappraisal of the head critical period", ***J. Insect Physiol.***, 44: 867-881 (1998).

23. Willott, E., Trenczek, T., Thrower, L.W., and Kanost, M.R.,"Immunochemical identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocytes types in *Manduca sexta*" ***European J. Cell Bio***., 65: 417-423 (1994).

24. Dushay, M.S. and Eldon E.D., "*Drosophila* immune responses as models for human immunity, ***Am. J. Hum. Genet.***, 62:10-14 (1998).

25. Dimarcq, J.L., Imler, J.L., Lanot, R., Ezekowitz, R.A., Hoffmann, J.A., Janeway, C.A. and Lagueux, M., "Treatment of 1(2)mbn *Drosophila* tumorous blood cells with the steroid hormone ecdysone amplifies the inducibility of antimicrobial peptide gene expression", ***Insect Biochem. Mol. Biol.,*** Oct. 27(10): 877-86. (1997).

Received:03.01.2002 *Accepted:31.01.2003*