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Establishment and characterizations of a new cell line from larval hemocytes of rose sawfly *Arge ochropus* (Hymenoptera; Argidae)

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Abstract
We established and characterized a hymenopteran cell line from *Arge ochropus* for the first time. The established cell line was named AOU-G, standing for *Arge ochropus* University of Guilan. This cell line was derived from larval hemocytes of the rose sawfly whose characteristics and susceptibility to 20-hydroxyecdysone have been verified. We also noticed four types of hemocytes such as prohemocytes, plasmatocytes, granulocytes and oenocytes. The population doubling time of 20th passage of this new cell line in EX-cell or Grace medium at 28°C was 3 and 5 days, respectively. Biochemical studies in rose sawfly hemocytes showed considerable changes in some component: protein, glucose, cholesterol and urea. Except urea, all components were significantly reduced. We suggest that the AOU-G cell line as a productive new cell line that could be very useful in studies concerning bio- insecticides, immunology, endocrinology and toxicology.
Keywords: *Arge ochropus,* Baculovirus*,* Cell culture, Ecdysone, Hemocytes

ایجاد و تعیین ویژگی‌های یک رده سلولی جدید از هموسیت‌های لارو زنبور برگخوار رز *Arge ochropus* (Hymenoptera; Argidae)

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چکیده
یک رده‌ی سلولی جدید از سلول‌های خونی لارو زنبور برگ‌خوار رز، *Arge ochropus* ایجاد شد. این رده سلولی براساس نام University of Guilan *Arge ochropus*، AOU-G نام‌گذاری شد. این رده‌ی سلولی از سلول‌های خونی لارو زنبور برگ‌خوار رز، به منظور ارزیابی خصوصیات و حساسیت به اکدایزون مورد بررسی قرار گرفت. ویژگی‌های شکل‌شناسی سلول‌های آن با استفاده از میکروسکوپ‌های نوری بررسی شد. از نظر ریخت شناسی در کشت اولیه سلول‌های خونی، چهار نوع سلول مشهود بود که به‌طور عمده شامل پروهموسیت‌ها، پلاسماتوسیت‌ها، گرانولوسیت‌ها و انوسیتوئیدها بود. زمان مضاعف شدن جمعیت این رده سلولی در بیستمین کشت مجدد در دمای ۲۸ درجه‌ی سلسیوس روی محیط کشت Ex-cell 400 و گریس به‌ترتیب ۳ و ۵ روز بود. مطالعات بیوشیمیایی روی کشت اولیه ناشی از سلول‌های خونی در محیط کشت Ex-cell 400 و گریس، نمایانگر تغییرات قابل ملاحظه‌ای در برخی از ترکیبات نظیر گلوکز، پروتئین، اوره و کلسترول در محیط کشت بود. به غیر از اوره که افزایش معنی‌داری را در محیط کشت نشان داد، بقیه ترکیبات اندازه‌گیری شده کاهش چشمگیری پیدا نمودند. ردهٔ سلولی AOU-G را به عنوان یک ردهٔ سلولی جدید معرفی می‌کنیم که می‌تواند در مطالعات مربوط به زیست‌شناسی، ایمونولوژی، غدد درون ریز وسم‌شناسی بسیار مفید باشد.
واژگان کلیدی: زنبور برگ‌خوار رز، باکولوویروس، کشت سلول، اکدایزون، هموسیت

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Introduction

Rose sawfly, *Arge ochropus* (Gmelin) (Hymenoptera; Argidae) is the most important defoliater of Rosaceae family flowers (Smith,1989). This pest causes drastic damages at larval stages; initially they feed in groups on flowers and on parenchyma of leaves, but later segregate and continue feeding on host leaves, individually. Terminal branches remain leafless and considerable damage is exerted to flowering organs. The damage caused by the adults is due to their typical way of oviposition, in fact the female cause significant damage to plants by laying eggs in tissues of young branches. They are insects with gregarious behavior. Therefore, in the case of severe infestations, the damage can also be severe for the economic loss they cause (Khosravi *et al*., 2015).

Insect cell culture technology has become an important tool in modern experimental biology and has been widely used in agriculture, medicine, and various fields of biology (Granados *et al*., 2007; Smagghe *et al*., 2009; Meng*et al*., 2017). The successful establishment of the first insect cell line by Gaw in 1959 and the subsequent continuous culture of four ovarian cell lines from *Antheraea eucalypti* (Scott) by Grace in 1962 significantly promoted a strong interest in establishing insect cell lines (Gaw *et al*., 1959; Grace, 1962; Smagghe *et al*., 2009). Today, more than 500 cell lines have been established from over 100 species of insects worldwide. The main sources of these insect cell lines include various tissues from the insects in the orders of Lepidoptera, Diptera, Coleoptera, Orthoptera, Hemiptera, Blattaria, Hymenoptera, Orthoptera etc. (Zheng *et al*., 2014). Also insect cell lines have become more important as a tool for the production of insect baculovirus expression vector (Granados & McKenna, 1995; Monteiro *et al*.,2017). Cell lines originating from different insect species tend to differ in their capacity to produce virus or express recombinant proteins (Sequeira *et al*., 2017). In addition, some lines are used for investigating specific virus pathogenic mechanisms in basic researches (Blissard, 1996). Therefore, there is a need to develop additional insect cell lines as a substrate for producing baculoviruses or recombinant baculoviruses and expressing baculovirus proteins (Iwabuchi, 2000). Although the damage caused by hymenoptera-associated viruses is increasing globally, only limited and poorly developed methods are available to treat these viruses. This may reflect the lack of a good cell culture system for hymeopterans. Several hymenoptera cell culture methods have been reported (Bergem*et al*., 2006; Hunter,2010; Goblirsch *et al*., 2013), but these methods have varied widely in their utilized target tissues, growth media, and isolation methods.

Sudeep *et al.,* (2002) established a cell line that was derived from the larval hemocyte of *Helicoverpa armigera*. These cells were characterized morphologically and cytogenetically and showed notable picularities in their growth pattern and cell shape. The

AFKM-On-H cell line was derived from hemocytes of the European corn borer *Ostrinia nubilalis* (Hübner) and demonstrated to be responsive to 20-hydroxyecdysone (20-HE) and some ecdysone agonists (Belloncik *et al.,* 2007). In addition, Muller *et al.,* (1999) studied a hemocyte-like cell line established from the malaria vector *Anopheles gambiae* expresses six prophenoloxidase genes. This cell line most likely derived from a hemocyte lineage, and represents an appropriate *in vitro* model for the study of the humoral and cellular immune defenses of *A. gambiae*. In our laboratory, a new cell line was established from the larval hemocyts of *A. ochropus* and was so named as AOU-G pertaining to initials of *Arge ochropus* University of Guilan. This cell line was found susceptible to 20-HE. We further characterized this new cell line and are being described in this report and observed changes to the hemocytes and cell immune response that suggest 20-HE negatively impacts this cells.

Material and method

Insect rearing

The fifth instar larvae were collected from infested rose shrubs in Rasht, Guilan province, north of Iran during the summer of 2017. They were reared in a growth chamber in controlled condition (24±1°C, 16:8 LD and 75±10% RH) on fresh rose leaves. The adult sawflies were placed in wooden cages of 40×20 cm and were provided with fresh rose branches for egg laying. The hatched larvae were transferred to new jars and fed with fresh leaves.

Primary Culture and Subculture

Surface of each larva was sterilized with 70% alcohol and dried with absorbent tissue paper. The larval hemolymph was taken with a 27-ga syringe by cutting one of the prolegs or by puncturing the posterior abdominal region with a very fine needle.

The blood was dropped onto a cultural flask that coated at the bottom with small amounts of Carlson's fluid (Carlson, 1946). Most of hemocytes settled on the bottom of the flask within 15 min. The cells attached to flask were washed by changing Carlson's fluid three times. The final washing was replaced with a culture medium. Culture media for primary culture, Ex-cell (EX-CELL® 420 serum-free medium, Sigma-Aldrich) and Grace (National cell bank, Pasteur institute of Iran) media were used, respectively. The culture media were replaced every 4 days. The FBS concentration was decreased from 20% to 15% at the 10th passage and from 15 % to 10 % at the 20th passage. The cells were observed under an Olympus BH2 inverted phase-contrast microscope (20x Magnification).

The first subculture was made when cells in the culture flask became confluent. Subculturing was carried out by detaching the cells from the surface of the flask, and

transferring into a new flask. The cells were suspended by flushing the medium over the monolayer using a Pasteur pipette. Hemocytes were categorized based on cell size, cell shape, and granularity (Söderhäll & Cerenius, 1992).

Maintenance and Characterization of Cells

The cultures were maintained at 28°C by renewing the medium once a week. Morphological studies carried out at passage 15. The growth curves were determined according to the cell density average. The cell population doubling time was calculated according to the method of Hayflick (1973).

Biochemical analysis

Concentrations of biochemical compounds were determined by enzymatic methods pursuant to the following protocols:

Glucose

The residual glucose concentration in the culture supernatants was measured as described by Siegert (1987). This assay was done by glucose assay kit (Biochem Co., Tehran, Iran). The absorption was read at 492 nm.

Protein

Protein was measured based on Bradford's method (Bradford, 1976) and by utilizing a total protein assay kit (Biochem Co., Iran). In this method, proteins made a complex purplish blue with an alkaline copper solution, which with its absorption value at 540 nm has a direct relation to the amount of the whole body protein.

Cholesterol

To measure the cholesterol, Richmond (1973) method was used. The principles of this method are based on hydrolysis of cholesterol esters by cholesterol oxidase, cholesterol esterase and peroxidase.

Urea

Urea was measured with urease-GDH kit. (Chem enzyme Co., Iran). In this method, ammonia ion is produced by urease enzyme and second reaction was catalyzed by glutamate dehydrogenase. Finally, reducing absorption rate was calculated at 340 nm.

Susceptibility of the AOU-G cell line to 20-hydroxyecdysone (20-HE)

The (20-HE) was donated by Professor K.Slama of Institute of Entomology Praha, Czeh from a plant source. The 20-HE was used in four different concentrations (0.375, 0.75, 1.5

and 3 µg/ml in DMSO). All compounds were dissolved in freshly prepared DMSO, and 1 µl of the solution was added to the test flask when adequate cell growth was reached in the primary cultures after two weeks. In this cell density level, about 70% of a cultural plate were covered with cells (about 4×10^5 cells/ml). Appropriate solvent controls (up to 3 µl per flask) produced no detectable effect on the cells. All the test cultures were monitored daily, for changes in cell morphology and number and compared with the solvent control.

Statistical analysis

Data obtained from the experiments were subjected to analysis of variance (ANOVA) and Tukey's student range test, at the significance level α=0.05 (SAS Institute 1997).

Result and Discussion

Establishment of the AOU-G cell line

Primary culture initiation

A new cell line has been established from larval hemocytes of *A. ochropus.* Obtaining primary cultures from hemocytes was successful using Ex-cell and Grace media. The cell number gradually increased, and some cells were deattached when the cell confluence reached 80 %. The suspended cells were harvested and then recultured in 3ml Ex-cell and Grace media supplemented with 20% FBS. The AOU-Gline was successfully sub-cultured after 15 and 21days of culturing in Ex-cell and Grace media, respectively (Fig. 1, 2). The interval time for subculture at first five passages were about 12 days at a ratio of 1:2, then became separate about and 9 days. The morphology of the majority of cells was round, with a spindle shape that became round at 80 % confluence. To date, the cells were successfully subcultured for 20 passages at an interval of 7 days.

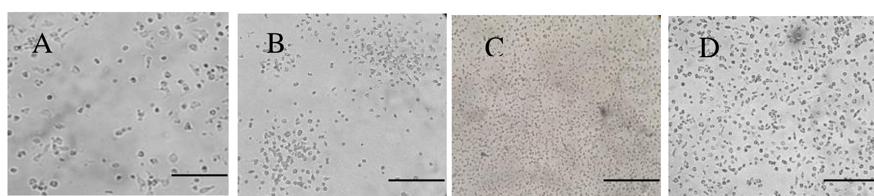


Fig. 1. Micrographs of the primary culture derived from larval hemocytes of *A. ochropus* in Ex-cell culture medium. (A) Primary culture observed at 1 day after culture initiation, (B) Cells in primary culture after 3 days of culturing, (C) after 10 days of culturing, (D) after 15 days of culturing. The scale bar is 20 µm.

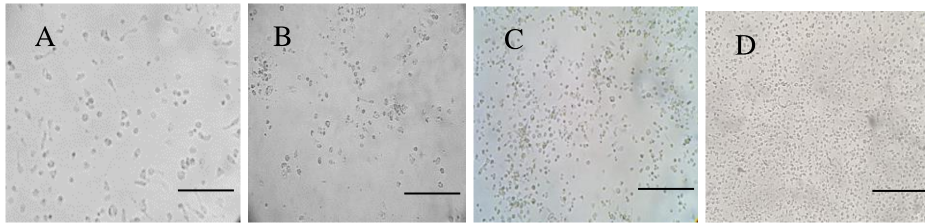


Fig. 2. Micrographs of the primary culture derived from larval hemocytes of *A. ochropus* in Grace culture medium. (A) Primary culture observed at 1 day after culture initiation, (B) Cells in primary culture after 5 days of culturing, (C) after 14 days of culturing, (D) after 20 days of culturing. The scale bar is 20 µm.

Right after setting up the primary culture, four types of hemocytes were distinguished. They were prohemocytes, plasmatocytes, granulocytes, and oenocytoids (Fig.3). Giannoulis *et al.,* studied both plasmatocyte forms and the granular cells comprise the majority of immunoreactive hemocytes of *Malacosoma disstria* (L.) larvae adhering to glass slides and oenocytoids have not been reported previously. When prohemocytes and plasmatocytes multiplied, they formed cell sheets. These cell sheets mostly consisted of an aggregated prohemocyte-like cells. These cells were small, spherical and highly refractile when viewed with phase contrast microscope. These cell-aggregates increased their size and liberated suspending cells. Overtime, these suspending cells multiplied actively and covered large area of culture flasks. Keddie *et al.,* (1995) reported that in the primary culture,cell population consist of the numerous hemocyte types, were replaced by dividing cells dominated by two types. As cells became confluent, UA-Md203 and UA-Md210 lines displayed cell morphology reminiscent of prohemocytes, although UA-Md210 were larger than UA-Md203 cells. Jones (1959) found that prohemocytes have been considered as stem cells which can be converted to some of the other hemocyte types.

Growth analysis

The cell lines were passaged in Ex-separate cell and Grace media, supplemented with 15 % FBS. Growth curves in these media are shown in Fig. 4. The cell growth was the fastest in Ex-cell medium and population doubling times were about 3 and 4.1 days for Ex-cell and Grace media, respectively. The doubling times calculated (Sudeep *et al*., 2001) were 3.9 d and 2.5 d for P8 and P18, respectively, for cell line from larval hemocyte of *Spodoptera litura* (F.). Also Keddie *et al.,* (1995) studied establishment and characterization of three *Malocosoma disstria* cell lines. They reported that both UA-Md210 and UA-Md203 cell lines doubled in approximately 50 hr, while UA-Md221 cell line doubled in 70 to 90 hr.

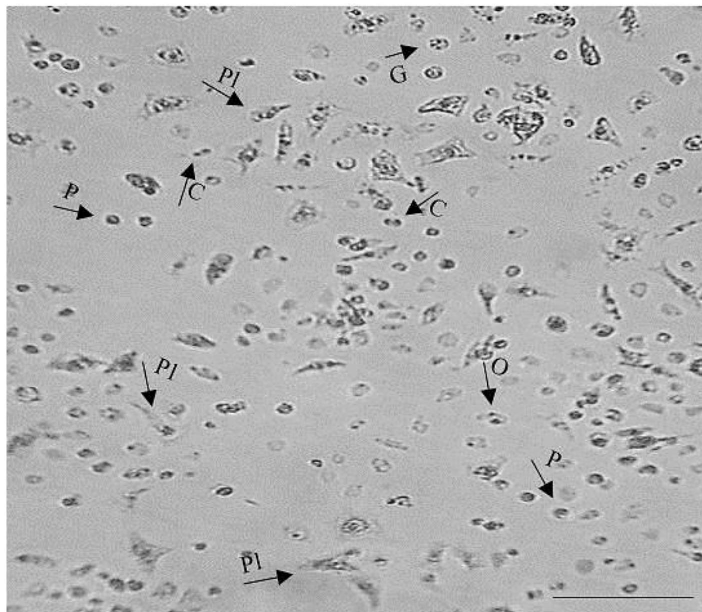


Fig. 3. Phase-contrast micrograph of AOU-G in primary culture, showing four major cell types, prohemocyte (P) plasmatocytes (Pl), granulocyteand (G) and oenocytoid (O). Circulating hemocytes dividing by mitosis (C). The scale bar is 20 µm.

The effect of Ex-cell and Grace media on biochemical changes in larval hemocytes primary cultures of *A. ochropus*

Measuring biochemical compounds was performed to understand what molecules were absorbed into the cells. The formulation of culture media can play a significant role in the production of virus or recombinant proteins in insect cells. Therefore, nutrient utilization and by-product accumulation were measured in larval hemocytes primary cultures of *A. ochropus* to develop a better understanding of cell metabolism. The utilization of glucose, total protein and cholesterol and production of urea were measured in primary cultures grown in Ex-cell and Grace media.

Glucose consumption profiles in Ex-cell and Grace media containing larval hemocytes primary cultures of *A. achropus* are shown in Fig. 5 (A) ($F = 28.23$, $df = 3$, $p < 0.0001$). Residual glucose in control vessel was 5.27 mg/dl, while its amount decreased considerably in vessel of Ex-cell medium and Grace medium containing larval hemocytes primary cultures of *A. ochropus*. This shows the increased metabolism of the cells. This is completely evident in cholesterol consumption and also the amount of urea produced as a by-product.

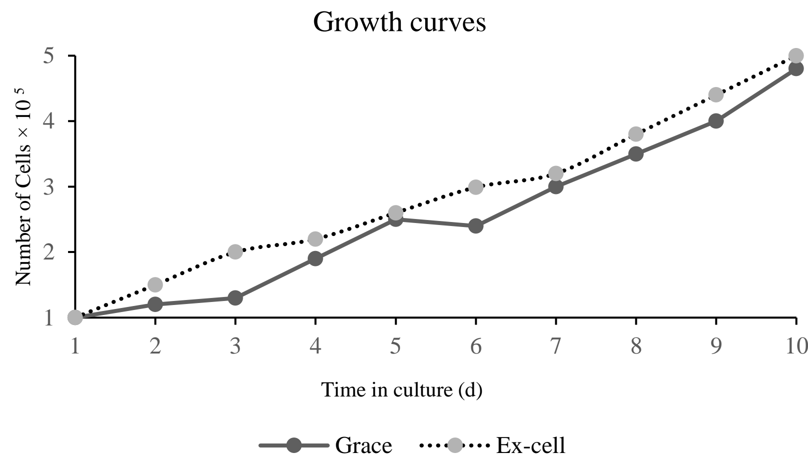


Fig. 4. Growth curves of AOU-G cell line in Ex-cell medium and in Grace medium supplemented with 15% FBS.

Our results on glucose consumption were consistent with those of Matindoost *et al.*, (2008) on *Bombyx mori* L. cell line. Their experiments showed that glucose was completely exhausted in primary cultures of *B. mori* embryonic tissue grown in TC-100 medium containing 10% fetal bovine serum (FBS) approximately 72 h post-infection with *Bombyx mori* nuclear polyhedrosis virus (BmNPV).

With a brief look at Fig. 5 (B), which is related to total protein levels in the spent media, it can be observed that total protein has decreased significantly in the cultures, while its level has been 2.4mg/ml in control medium ($F = 41.55$, $df = 3$, $p < 0.0001$). This could be due to the break-down of proteins into amino acids and their entrance into TCA cycle as keto acid (Nath *et al.*, 1997).

The cholesterol levels have shown a diminution in the Ex-cell medium ($F = 1.76$, $df = 3$, $p < 0.2320$) (Fig. 5) (C). No considerable variation is observed among treatments compared with the control. Authors speculated that lipid catabolism is possibly contributing to the energy supply (Matindoost *et al.*, 2008). The nutrient requirements of insects are generally similar to those of vertebrates, with the exception that their diet must contain a source of cholesterol. Insects have no capacity for steroidogenesis. Cholesterol is used for the formation of cell membranes and for the synthesis of important steroid hormone 20-hydroxyecdysone (Law &Wells, 1989). Cho *et al.*, (1989) reasoned that cholesterol might play a vital role for the growth of insect cells and viral replication because it is common in the synthesis of cell membrane and is particularly essential in the synthesis of the growth and development hormone, ecdysone.

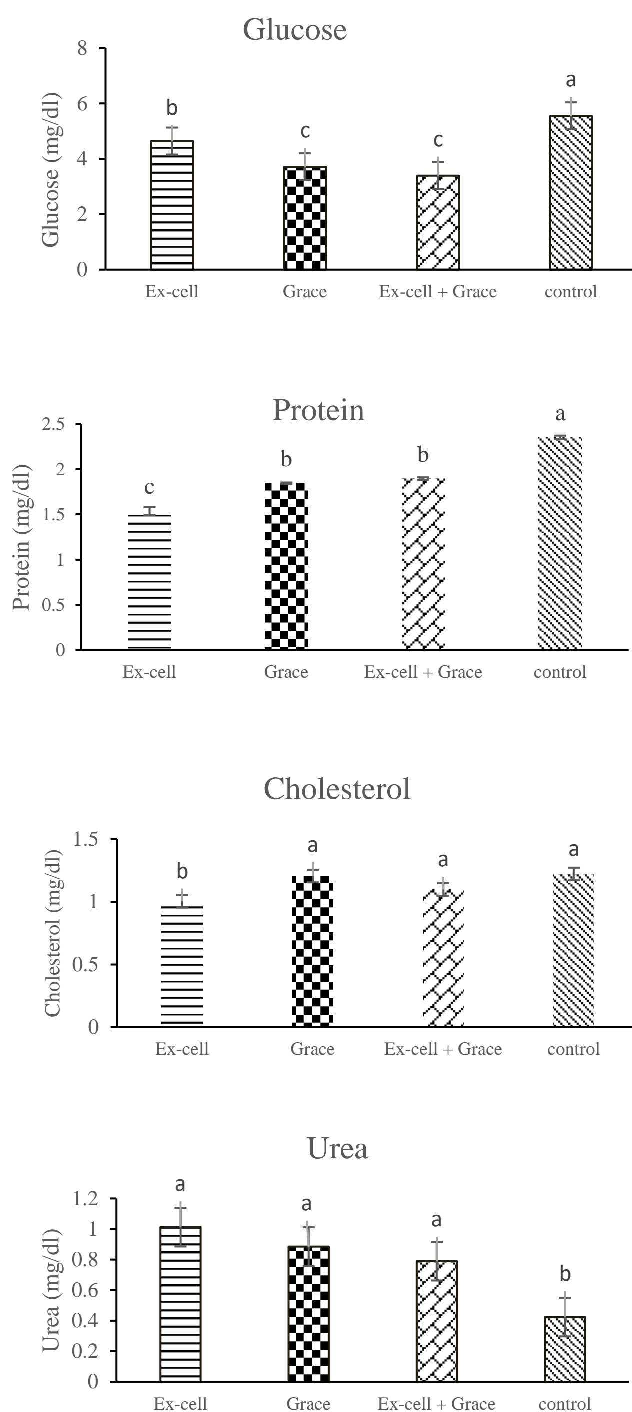


Fig. 5. The changes of glucose, protein, cholesterol and urea in different primary cultures containing of *A. ochropus* hemocytes (mean ± SE). There is no significant difference between the numbers that are shown with the same letter ($P<0.05$).

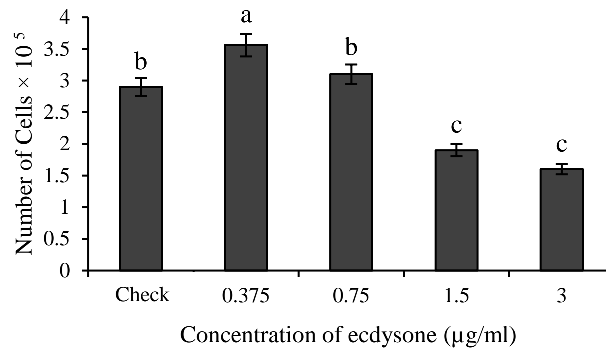
Urea concentration in the spent media of cultures showed significant difference (F = 44.81, df =3, $p< 0.0001$) (Fig. 5) (D), in a way that from 0.36 mg/dl in the control, it had increased to 1.02 mg/dl in the Ex-cell medium and 0.98 mg/dl in the Grace medium. However, these differences are not considerable among three different media containing larval hemocytes. Amounts of urea in the culture medium depend on the reactions taking place after protein metabolism; in fact they are the excreted end product of hemocytes. Hence, their amount is correlated with the amount of protein in the cells. This is consistent with the results obtained by Matindoost *et al*., (2006) on *B. mori* embryonic primary cultures, which showed increased amount of urea in the spent medium.

Ecdysone action on AOU-G cell line

The changes in cell number and morphology in response to treatment with α-ecdysone were repeatable and readily observed. We were able to establish dose-response relationships with a maximum response in concentration of 3 µg/ml (F = 41.39, df = 4, $p< 0.0001$) (Fig. 6), a range similar to that found necessary to produce morphological changes in *Aedes albopictus* mosquito cell line(Fallon *et al*., 2010).Also Muller *et al.,* (1999) studied 20-hydroxyecdysone effects on prophenoloxidase (PPO)expression in cell line 4a-3B from the malaria vector *A. gambiae,* the transient induction of the larval PPOs following a blood meal suggested the possibility of hormonal regulation by the moulting hormone 20-HOE, however this experiment confirmed this supposition and revealed that the effects of the hormone on the cell line are gene-specific, paralleling the effects of a blood meal in the intact mosquito. Understanding the immune responses and the immune potentials of this insect can be established and hence the efficiency of foreign agents to overcome the immune system of these insects could be incorporated.

Conclusions

The technology of cell culture is of immense important for various applications including baculovirus biotechnology. A new cell line derived from the larval hemocytes of *A. ochropus* was established and characterized in this study. It is important to have a larger panel of hymenopteran cell lines, as they may serve as efficient tools to advance studies in multiple areas of biology and biomedicine, including immunology, endocrinology, toxicology, biochemistry, parasitology, evolutionary and biology. In addition, the method of obtaining hemocyte from haemolymph in this report may prove useful in initiating cell lines from other Hymenoptera.



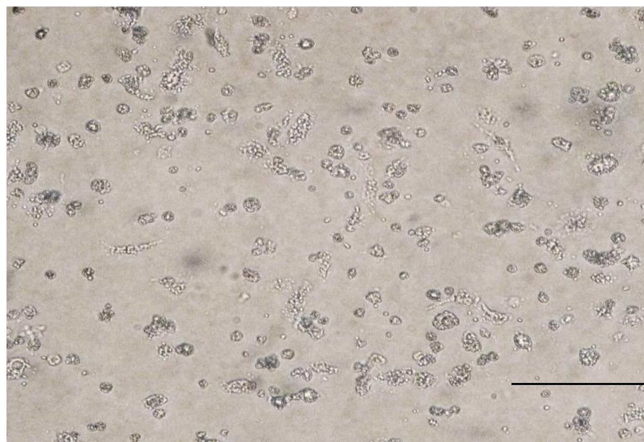


Fig. 6. The changes in cell number and morphology after 168 h in response to α-ecdysone. Bar = 20 µm (phase contrast).

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