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باشراف

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ALI

Certification

We certify that the preparation of this thesis entitled "*In vitro maturation and phenotyping of coelomocytes and gonad cells using different culture media and additive hormone*

in sea urchin paracentrotus lividus" by the student " *Ali abdul-hussain ghazzay*"

was prepared under our supervision at the Department of Biology, College of Science, University of Baghdad, as a partial fulfillment of the requirement for the degree of **Doctorate of Philosophy (Ph.D) in Biology/cell biology .**

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BrdU	Bromodeoxy uridine
DAB	Di-amino-Benzoidine
PBT	Phosphate buffered tween
PBS	Phosphate buffered saline
NGS	Normal goat serum
FCS	fetal calf serum
FBS	Fetal bovine serum
EDTA	Ethylene diamine traacetic acid
TEM	Transmission electron microscope
L-15	Leibovitiz medium
MEM	Minimum essential medium Eagle
E_2	17β oestradiol

ABSTRACT

The study was aimed to developing appropriate media for coelomocytes and gonad cells culture of sea urchin *paracentrotus .lividus*. The effect of different media: L-15, MEM, M199 have been investigated for their influence on the viability of primary cell cultures. Modified Leibovitz L-15 medium supported the best survival of coelomocytes and gonad cells in *vitro* primary cultures. While Coelomocytes and gonad cells from the sea urchin. were maintained for more than 14 days in Leibovitz L-15 medium, and 9 days in MEM, M199 only.

The Results pointed out the positive effect of oesteradiol (E_2) combined with cultures media, on the viability of coelomocytes which prolonged up to two weeks in L-15 and more than 12 days in MEM, M199 medium respectively.

Cell cycle activity was investigated by incorporation of the thymidine analogue; bromodeoxyuridine (BrdU) labeling.the results showed that short-term exposure to this substance induced cell proliferation of coelomocytes.

Density gradient step centrifugation with Iodoxinol was used to separate coelomocyte populations into four subpopulations enriched in amoebocytes, colorless spherule, red cells and vibratile cells.

It was concluded that primary cultures resulted in good adherence of coelomocytes in L-15medium and produced lamellipodia/filopodia to adhere the substrate.

INTRODUCTION

The establishment of primary cell culture from sea urchin *Parcentrotus lividus* is particularly attractive because of its many potential applications in a

wide range of fields. Sea urchins have long been an important model organism for studies in developmental biology and embryology, have also recently become of particular interest to pharmacology due to the biologically active quinine compounds and pigments produced by several species (Dixon *et al*., 2002).

Sea urchins were also emerging as an important aquaculture species, necessitating the establishment of cell lines to study and develop treatments for diseases that could plague aquaculture operations. Attempts also have been made for establishing primary marine invertebrate cell cultures from a variety of source organisms including sponges, molluscs, crustaceans, urochordates, and echinoderms have been made repeatedly over the past four decades (Rinkevich, 1999; Moss *et al*., 2000).

The majority of the primary cultures in these experiments have exhibited short lifespan on the order of 4 to 6 weeks. Although there has been only one report on prawn *Penaeus. monodon* ovary primary cultures remaining alive in excess of 12 weeks (Gago *et al*., 2001). Moreover, all invertebrate primary cultures have consistently displayed very low proliferative levels with cell degeneration dominating over proliferation. Additionally, all attempts at sub- culturing from these primary cultures have proven unsuccessful (Bulgakov *et al*., 2002).

As such, continuing research in this field has focused on isolating tissue sources with the highest potential for long-term cultures, optimizing the efficiency of the culture methods and identifying media supplements capable of supporting long term culture of echinoderm cells. Selecting tissue source s with a high capacity for proliferation and minimal risk of microbial contamination is the primary starting point for long term echinoderms cell culture(Moss *et al* .,2000)

In the case of sea urchins, coelomocytes have been widely used because of their abundance, ease of collection, and inherent immune functions that minimize microbial contamination. However, despite the potentially long lifespan of coelomocytes in culture, high levels of coelomocyte growth and proliferation *in vitro* have yet to be documented (Cornet, 2000).

A variety of other source tissues have also been experimented with in order to select cells that have a higher inherent capacity for cell division with less fastidious requirements and more robustness under sub-optimal conditions. In particular, reproductive tissue has been used in several studies. Ovarian explants from the prawn *Penaeus monodon* have been reported to remain alive in culture for more than 12 weeks (Dormat *et al*., 2004). Similarly, embryonic tissue has been demonstrated to be mitotic in culture and has been used in several studies as a cell source for long-term culture and culture substrate selection experiments (Parma *et al*., 2006).

Particularly, in this research a special focusing were made on oestradiol (E_2) and its possible physiological role on echinoid coelomocytes from the coelomic fluid of the echinoid *Paracentrotus lividus*. The effect of three different concentrations of the hormone on cell viability was tested *in vitro*. In addition, this is the first report of the development of primary cell cultures from gonads of the edible sea urchin *P. lividus* using Leibovitz L-15, MEM and M199 media. Little success was achieved with M199 and MEM media. The efficiency of Leibovitz's L-15 medium for promoting growth and cell survival of invertebrate tissues was confirmed.

The most relevant outcomes of the present study:

- Maintaining in viable conditions primary cell culture of *Paracentrotus lividus*, with different cultures media.

- Setting up improved methods to produce primary cell cultures particularly addressed to: increase the cell amount *in vitro*, decrease contamination due to bacteria and/or mould, eliminating undissociated tissues/skeletal parts, and obtain indeed a pure cell suspension.
- Characterizing phenotypes for coelomocytes *in vitro.*
- Giving evidence of cell activities such as cell adhesion processes on substrate, particularly developed by echinoid coelomocytes, and production of lamellipodia/filopodia.

2-1-Primary Cell Culture of Invertebrate.

It is well known that animal cells (from vertebrates) and plant cell, under *in vitro* conditions, were used in a variety of circumstances and in many scientific disciplines as important tools for experimentation .However, all attempts to develop cell cultures from marine invertebrate have been ineffective up to now in spite of the acknowledged need for cell cultures from invertebrate species that can be very important in aquaculture or in the pharmaceutical industry (Candia and Bonosora,2001 ; Rinkevich, 2004).

The large group of marine invertebrates, which encompasses more than 20 different phyla, represents a rich potential source of cell and tissue types that significantly differ from one group to another. Many cell types from a variety of invertebrates appear to possess extensive morphogenetic potential (Candia and Bonasoro, 2001a, b).

Unfortunately, the low mitotic activity of many tissues and the slow adaptation of the cells to culture conditions (up to 3-12 weeks) constitute a main huge limit to progress in obtaining continuous cell lines from marine invertebrates(Odintsova, 2005). Therefore, all previous efforts aimed at establishing permanent cell lines of these animals have so far failed. One less ambitious alternative for invertebrate was the development of methods for establishing of primary cultures .A number of media have been proved to support long-term cell survival (up to several months) in cultures derived from different tissues. In some cases, a mitotic activity was reported, but those cultures did not suitable results (Moss, 2000).

The lack of significant progress of echinoderms cell cultures may be related to an inappropriate comparison of echinoderms cell culture requirements with the culture conditions of invertebrate cell lines or may be due to special or unique requirement needed for echinoderms cell cultures (Dixon *et al*., 2002; Parma *et al*., 2006).

On the other hand, it is clear that echinoderms cell cultures require unique approaches and bear obstacles not recorded in invertebrate cell cultures. Some of these points (aseptic conditions, media supplements. substrate cell types , selection of species, selection of basic media and additives and the merit of feeder layers (Rabinotiz and Rinkevich, 2003).

Primary cultures of invertebrate cells during the last 10 years concentrated on a variety of cellular differentiation aspects. These Studies were performed on sea urchin cells (Mothersill, 2000), primary shrimp cultures (Dormart *et al*. 2004) and cultures from sea cucumbers, following cytogenetically applications (Custotdio *et al*., 2004)

There were two characteristics of primary marine invertebrate cell cultures which were not recorded as important in most other invertebrate cell cultures.

(1) The impact of substrates on cellular differentiation, for example, two different cell types (epithelial or mesenchymal) may be developed(1 week) after initiation of cell cultures from sea urchin *Paracentrotus* . *lividus* larvae at the gastrula stage . Epithelial cells predominated wells coated with polylysine (with a high level of [3H]thymidine incorporation), while the addition of fibronectin or an ascidian) resulted in syncytia of different shapes and sizes and conglomerates typical oncoprecipitin A (onco A, as a specific glycoprotein isolated from of primary mesenchyme cells) were formed(Zhao *et al*., 2003).

(2) A variety of cell types in marine invertebrate tend to form aggregates and, or monolayer syncytia (Thorndyke *et al*., 2001). As a result of the limited number of studies aimed at the production of continuous cell culture from echinoderms, there were almost no data for the response of echinoderm cells in long-term conditions. Dissociation protocols, media, general conditions and organs to be used as a source for culture requirements to be defined in future experimentation (Wilkie, 2001).

Moreover, all attempts to develop long-term, continuous cell cultures from marine invertebrate have not been rewarding and this field was still in its preliminary stages, and there was one promising approach which has proven to be valid in several studies: the target organ, cell source for *in vitro* studies. were obtained from invertebrate epithelia (gills, mantle, pharynx,buds,outer epithelium), muscles (heart, a variety of other muscles), neurons, blood (hemolymph), internal organs (glands, hepatopancreas), gonads and others. Two of the most promising approaches were the amenability of larval, embryonic cells and the use of organ culture in a spontaneous dissociation protocols (Thorndyke and Candia, 2001).

The use of larval and embryonic cells *in vitro* has been employed in a variety of fresh water and marine invertebrates, including echinoderms, sponges and crustaceans. In the invertebrates, embryonic cell lines, which were restricted to insects, showed their amenability as an excellent tool for a variety of approaches. This source of cells is also less vulnerable to contamination from bacteria, yeast and mold (Vickery, 2001).

This is of further importance because available tissue sources from marine invertebrate, particularly those that contain intracellular micro-organisms and crypto-organisms which cannot be completely sterilized. Moreover, when compared to adult primary cultures, embryonic cultures survived longer and in many cases, consistently yielded cultures that were capable of at least limited growth *in vitro* (Muller *et al*., 2000).

This fact may provide an obstacle in characterization of cellular components developed *in vitro* from dissociated larvae and embryos. On the other hand, larvae or embryos may provide large numbers of synchronously developing cell lineages and possess cell populations with high mitotic indices which were better candidates for primary and continuous cultures than any other organ or tissue (Oji, 2001).

Organ cultures and spontaneously dissociated tissue fragments were two approaches which employed similar protocols for two different purposes. long-term cultivation of tissue fragments which consist of foci for cell proliferation that migrate from the explants into the medium during culture, and short-term cultivation of tissues that dissociate spontaneously (without the employment of any chemical or enzymatic treatment) and provide a wide scale of cell types for *in vitro* applications(Freshney, 1994).

Organ culture for sustained viability of proliferation of cells was mainly carried out on solitary tunicates as cultured pharyngeal fragment sites of hematopoiesis were used (Parma *et al*., 2006). In this system, pharyngeal

tissues remained viable and proliferated. This maintained the pool of hemocytes within the explants and facilitated the migration of hemocytes from explants into the culture medium (Oweson *et al*., 2008).

The same methodology has been employed for establishing primary epithelial cultures from tunicate buds ,cells spread out from the epithelial explants and proliferated (at least for a short period of 1–2 weeks) on the substrate. Spontaneous dissociation was based on inserting tissue fragments or whole larvae into tissue culture medium, where they were dissociated without employing any chemical, enzymatic or further mechanical treatment. This was successfully performed on shrimp and bivalves cell cultures (Bayne, 1998).

Cultures of cells from explants were superior to cultures obtained from dissociated tissues and provided viable cells, sometimes even more cell types. This methodology may be explored further to find additional improvements which will reduce stress to cells during tissue dissociation (De Rosa *et al.*, 2003).

In case of evisceration processes it was possible to establish a correlation between the stages of regeneration and the cell growth potential in culture. Only cells of primary intestinal cultures, obtained (14–16 days) after evisceration, were involved in active proliferation; the number of cells doubled by the 20th day of culture. (Candia *et al*., 2009).

Two main cell types (presumably, enterocytes and coelothelial cells) were present at the start of the primary cultures. The proliferative activity in culture not only appeared to depend significantly on the stage, but also on the specific substrate used.When seeded and cultured on poly-lysine substrates,

some cells spread and grew as a multilayered mass; these cell sheets increased significantly in size within 2 weeks (Cornet, 2000).

In vitro studies on cells were focused on coelomocytes and gonad and muscle cells with few investigations on neural cells based on investigations concerning neural tissues of other marine invertebrate(Dixon *et al*.,2002).

A major difficulty was the frequent contamination with protozoa, bacteria and fungi that causes loss of most cultures. Most authors also point out the lack of data on the *in vitro* physiology of the cells and the resulting absence of appropriate culture media and conditions to explain the low frequency of mitosis observed. Cells were maintained in nutritive culture media to which supplements were added. Except for a few trials with natural or artificial seawater), most culture media consist of balanced saline solutions supplemented with carbohydrates and amino-acids; Antibiotics were frequently added in order to prevent microbial contamination. A first strategy was to make media *de novo,* the aim being to accurately mimic the composition of hemolymph of the bivalve or that is put into culture (Chen, 1999).

A second strategy was to derive them from commercial media sold for vertebrate cell cultures and modified through addition of mineral salts in order to raise their osmolarity. As results obtained so far showed no difference between *de novo* made media and modified commercial media, most researchers now choose the second strategy. The Commercial media most frequently tested and used were Eagle's MEM, Hank's 199; PRMI 1640 has been tested and used (Fraser and Hall, 1999).

Leibovitz medium (L-15) has recently been reported to give good results. Various media supplement have been used: purified compounds, mainly vitamins, non-specific nitrogen homologous hemolymph, fetal calf

serum (FCS), horse serum, yeast or bacterial extracts, extracts prepared from whole animals or from specific tissues such as gonads and cerebral ganglia (Ducati *et al*.,2004).

Although echinoderm tissue could be maintained in culture under certain conditions, no enhancement of outgrowth was seen using conditioned media. The lack of effects by factors known to be active in invertebrate systems was not surprising or uncommon in Echinoderm studies (Kelve *et al*., 2003).

It was likely that the tissues require some specific native conditioning factor which is often found in neural or 'blood'-derived tissue (Dupont and Thorndyke, 2006). However, growth-related native factors were concentrated and purified (although did not appear always necessary), this might be required for outgrowth of echinoderm cell cultures (Hibino *et al*., 2006).

The only *in vitro* studies on echinoid, Primary cell cultures have been preliminarily obtained from the arm explants and arm regenerating tissues of *Antedon. Mediterranean* (Mozzi *et al*., 2004).

The echinoid model represents a good experimental model from developmental biology to molecular genetics issues (it was relevant that sea urchin genome was completely sequenced).

Although echinoids have no striking regenerative properties, regular sea urchin samples such as *Paracentrotus. lividus* have a large amount of coelomic fluid containing several cell phenotypes (coelomocytes, amoebocytes, phagocytes, vibratile cells and red cells), some of which contribute to repair/defense processes and were similar to those already characterized in other echinodermata(Pinsino and Thrndyke,2007).

Sea urchins *Paracentrotus. lividus* were an ideal candidates for biotechnological studies because of their ability to produce a broad spectrum of biologically active molecules (Hibino *et al*., 2009). Unfortunately, primary cell cultures, obtained from the coelomic fluid have a low proliferation level,

and cell degeneration predominates over proliferation. Therefore, all efforts addressed to obtain long-term cell lines of echinoids have so far failed. It is well established that in order to obtain a continuous animal cell line, tissues with high growth potential, such as embryonic or neoplastic tissues, should be used (Odintsova *et al*.,2005).

The experimental approach employing cell cultures of echinoderms models should be considered an appropriate and potentially powerful tool to address the wide problem of the biology of stem cells (Rinchevich, 1999).

2-*2*- Sea urchin*: Paracentrotus. Lividus*

In the present study we explored echinoderm cell culture by using the echinoid *P. lividus* as experimental model. This species is widely distributed in the Mediterranean Sea and in the North-East Atlantic Ocean, from Ireland to the Canarias (Gago *et al*., 2001). In the coasts it is one of the most common sea urchins, widespread in the lower rocky shore, in the shallow sub littoral areas and in beds of sea grass (Moschino and Marin, 2002).The high ecological relevance of this echinoid is related to its impact on the algal community, indeed *P. lividus* is one of the main Mediterranean herbivorous species on rocky bottoms and its foraging activity remarkably affects the composition and the dynamics of algal and rocky littoral pools (Kelly, 2001).

In addition it is a commercially relevant species since its gonad are an appreciated food in many Mediterranean regions (Huges *et al*., 2006) *P. lividus* is a regular sea urchin, belongs to the phylum Echinodermata and to the class Echinoid. It is a gonocoristic species, but some hermaphroditic specimens have been occasionally found (Laweence *et a*l., 2000); although sex determination mechanism is still unknown, most authors suggest it was on genotypic basis (Tilbrook *et al*., 2000).

It has a pentamerous radial symmetry and a globoid shape. The tests are composed of fused plates of organs calcium carbonate which enclose and protect the internal cells (Spirlet *et al*., 2000). Regular echinoids have five gonads, located at the aboral surface and attached and anchored to the inter-ambulacral plates by thin mesenteries of the peritoneum (Gago *et al*., 2001). Gonads have a sac-like elongated shape with an alveolar structure. Each gonad is composed of hundreds of acini opening into a central gonoduct; this latter reaches the gonopore, opening in one of the genital plates surrounding the anus. Mature gametes are shed through the gonoducts and gonopores directly to the sea water (Barbaglio *et al*., 2007).The interstitial cells of the gonad are commonly called nutritive phagocytes these somatic cells vary in both morphological aspect and chemical composition throughout the reproductive cycle (Goldstone *et al*.,2006).

At the beginning of gametogenesis, they are voluminous, with a diameter of up to 50µm, and occupy almost all the acinal lumen, surrounding the germinal cells with their long processes (Ben Besten, 2001). During the cycle they decrease in size, probably because of nutrient transfer to developing gametes, until they are crippled by mature gametes. Generally, they have a round nucleus, often characterized by an evident central nucleolus and big heterogeneous cytoplasmatic inclusions, made of glyco- and lipoproteins, polysaccharides, proteoglycans and lipids.

Nutritive phagocytes play multiple roles: they not only provide support, protection and nutriment to the growing germinal cells, functioning both as a storage and transfer site and an active synthesizing place, but they also “clean” the gonad after spawning (Huges *et al*., 2005).

The maturation of male germinal cells occurs, as in most of the animals, through a series of meiotic divisions and morph-functional

transformations during the process of spermatogenesis. Scattered primitive spermatogonia are present in groups at the base of the germinal layer during non-reproductive months.The spermatocytes are closer to the lumen of the testis than the spermatogonia and they form several layers which converge in spermatogenic columns infiltrating the nutritive phagocyte layer (Gago *et al*., 2001).

The following spermatid stage is characterized by several differentiation events, collectively called spermiogenesis, which converts the relatively undifferentiated, early spermatids into highly specialized,functional spermatozoa. Mature spermatozoa are accumulated in the centre of the acinal lumen. (Leoni *et al*., 2001).

As spermatogenesis, oogenesis consists of different progressive stages (oogonium, primary oocyte, secondary oocyte and mature ovum), each of them is characterized by several morphological and biochemical modifications. Oogonia are about 5-7 μm in diameter and have a large, oval nucleus with one big nucleolus, they are lined up in the close vicinity of the germinal epithelium base, often organised in cluster (Spirlet *et al*., 1998).

The most prominent feature of the young undifferentiated oocytes is the large nucleus (germinal vesicle). The nucleolus, which is in contact with the inner nuclear membrane in the oogonium, in the early vitellogenic oocyte is found in the centre of the nucleus(Silvia,2010).

In sea urchins meiosis is completed within the ovary and mature eggs are accumulated in the lumen of the acinus. Besides the plasma membrane and the vitelline membrane, a third transparent layer, called jelly coat, covers

the sea urchin eggs and plays important roles in fertilization (Walker *et al*.,2000).

The maturity stages of the gonadal cycle can be determined by histological analysis. In *P. lividus* previous studies have described a cycle composed of a variable number of stages (Silvia, 2010). On the basis of these works and previous studies we considered a cycle of five progressive stages.

***0-Spent*:** This is the stage after the spawning event. Spent ovaries have thin acinal walls and appear empty except for some relict oocytes. The number and type of vitellgenic oocytes and ova present in the ovary is variable and they will be eventually resorbed by nutritive phagocytes. The testes seem to be devoid of content although relict spermatozoa may be present. In both sexes nutritive phagocytes appear as a pale meshwork around the periphery of gonads.

***1-Recovery*:** Oogonia/spermatogonia and rare young oocytes /spermatocytes are the only germinal cells present along the gonadal walls. The very early stages of the gametes can make the identification of the sex very difficult. Nutritive phagocytes are big and full of heterogenic inclusions, including material phagocytosed from relict gametes.

***2-Growing*:** The gametogenesis processes have begun. The ovaries contain clusters of primary oocytes along the acinal wall and the testes periphery is lined with a thin layer of spermatogonia and primary spermatocytes. Nutritive phagocytes are full of nutritive material and form a regular meshwork all over the acinus.

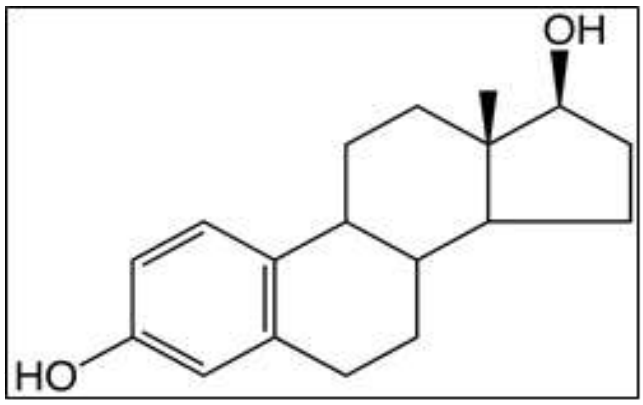
***3-Premature*:** Gametes at all stages of development are present in the gonad. Large primary oocytes start migrating towards the centre of the acinus and when they reach maximum size, they undergo maturation and first ova can start accumulating in the lumen. Nutritive phagocytes are still present, although displaced from the lumen by large oocytes. In testes columns of

spermatocytes can be observed along the acinal wall and mature spermatozoa begin to accumulate at the centre of the acinus. As in ovaries, nutritive phagocytes are displaced from the centre by the mature spermatozoa.

***4-Mature*:** At this stage ovaries are packed with mature ova. The nutritive phagocytes are few in number and confined to the border. Mature testes are packed with spermatozoa too and nutritive phagocytes are limited to the periphery. The spawning event occurs at this stage (Barbaglio et al., 2007).

2-3-. The hormone: 17β-Oestradiol (E_2).

17β-oestradiol (E_2) is a steroidal hormone synthesized from cholesterol. Together with estrone, it belongs to the class of estrogens (Lafont and Mathieu, 2007)



The role of estrogens in human reproduction was well-known. Estrogens are the most important reproductive hormones in women, responsible for the development of secondary sexual characters and the regulation of menstrual cycle (Lavado *et al*., 2005)

Overall, in vertebrates, estrogens regulate the metabolic, behavioural and morphological changes during the reproductive cycle of females, and they also play an important role in several processes of males. For example, in Eutherian females, beside effects on endometrium proliferation and cervical

mucus, E_2 is the only substance able to mediate oestrus symptoms. With enlargement of the developing follicle E_2 production rises and brings to ovulation. In males, it is involved in different reproductive activities, such as testicular fluid production or intratesticular sperm transport (Janer *et al*., 2005a).

The presence of estrogens and, particularly, of E_2 has been documented in almost all invertebrate groups but the complete demonstration for an endogenous synthesis is available only in molluscs where investigations concerning their biological effects have shown their direct or indirect connection with reproduction (Kohler,2007).

2-3-1-E_2 in Echinoderms.

The presence of E2 and, generally, of estrogens has been demonstrated in echinoderms (Varaksina and Varaksin, 2002). Most studies referred to asteroids and echinoids, where these molecules have been detected by several techniques, such as bioassay, radioimmunoassay and gas chromatography-mass spectrometry (GC-MS) (Roepke et al*., 2006).*

Several researches have also demonstrated the presence of vertebrate-like steroid metabolic pathways in different echinoderm tissues (Janer *et al*., 2005b). However, while the androgen metabolism has been successfully described (Wallace, 1999), the estrogens biosynthesis is still unclear and only few studies have investigated this pathway (Janer *et al*., 2005c).

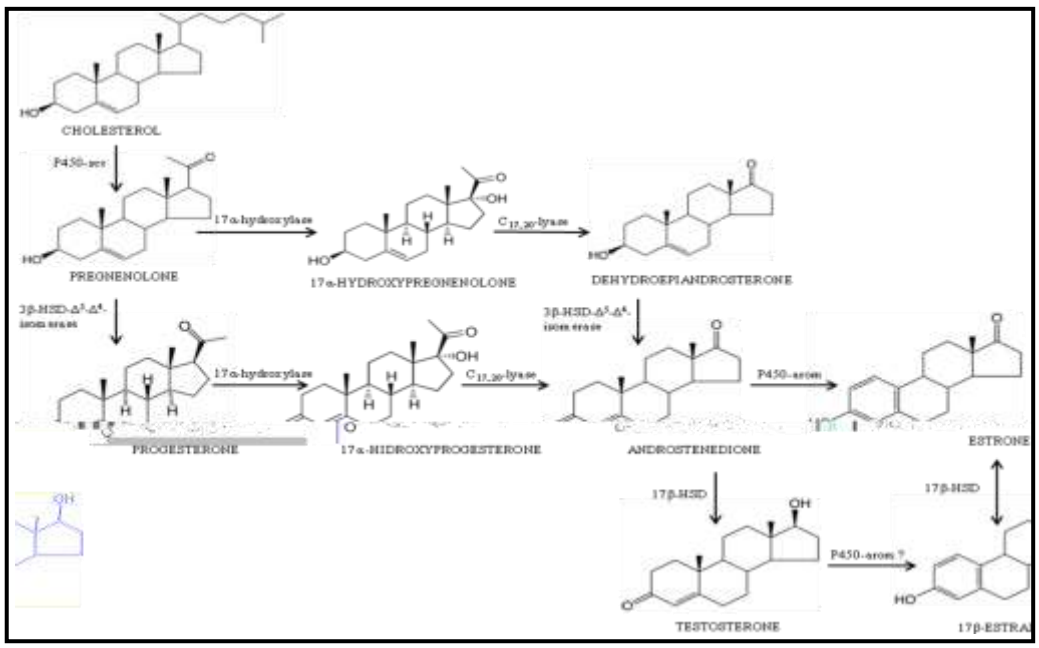


Figure (2-2): Schematic representation of steroid synthesis and metabolism studied in echinoderms. P450-scc: P450 side-chain cleavage; P450-arom: P450-aromatase; 17β-HSD: 17β-hydroxysteroid dehydrogenase (Silvia, 2010).

The conversion of E_2 into estrone occurred in sperm preparations of the echinoids *Echinarachnius. parma* and *Arbacia. punctulata* and both gametic and somatic tissues of the echinoid *Lytechinus .variegatus* showed the capacity to interconvert free estrogens (estrone and estradiol) *in vitro* (Lafont and Mathieu,2007). Although these results suggest the endogenous presence of estrogen metabolizing enzymes, such as the 17β-hydroxysteroid dehydrogenase (17β-HSD) in echinoderms the synthesis of E_2 from androgens has not been clearly demonstrated yet (Lange *et al*., 2003),figure (2-1,2-2).

At present, only few studies (Wasson *et al*., 200b) reported the presence of some-kind of aromatase (CYP19) activity, the enzyme responsible for androgens to estrogens conversion in vertebrates, i.e. the conversion of androstenedione into estrone and testosterone into 17β-oestradiol respectively. However, the echinoderm enzyme complex is likely to be structurally very different from the vertebrate aromatase since a

(CYP19) gene homologous has not been found in the completely sequenced genome of the sea urchin *Strongylocentrotus. purpuratus* (Martin *et al*.,2006).

In echinoids, Lavado *et al*. (2006) showed that this aromatase-like activity, particularly androstenedione–estrone conversion, occurs in both digestive tube and gonads of *Paracentrotus .lividus* but in the former the activity is up to 20-fold higher than in the latter. These data suggest that the digestive tube could be the main biosynthesis site of estrogens in this echinoderm class (Janer *et al*., 2005b), figure (2-3).

L. variegatus has the capacity to synthesize conjugated estrogens: esterified estradiol and aqueous-soluble estrogens. The accumulation of these conjugated estrogens indicates that these compounds are major metabolites of estrogen metabolism in sea urchins. The sulfation of steroids may inhibit their biological activity by decreasing their affinity for steroid receptors and increasing their rate of elimination (Martin *et al*., 2006). Sulfation reactions are catalyzed by sulfotransferase (SULF) enzymes which transfer the sulfuryl group from 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to an appropriate group (usually –OH) on the substrate (Coveney *et al*., 2001).

Active steroids may be regenerated from sulphate conjugates by the action of sulfatase enzymes which catalyze the reverse reactions. Fatty acid conjugation (or esterification) renders steroids to a polar form, which is retained in the lipoidal matrices of the body, while reducing their activity, bioavailability, and susceptibility to elimination (Lavado *et al*., 2006).

Esterification reactions are catalyzed by fatty acid acyl-CoA acyltransferase enzymes and their products, steroid esters, do not bind receptors but they can be hydrolyzed by esterases liberating again the active steroid (Wallace, 1999).

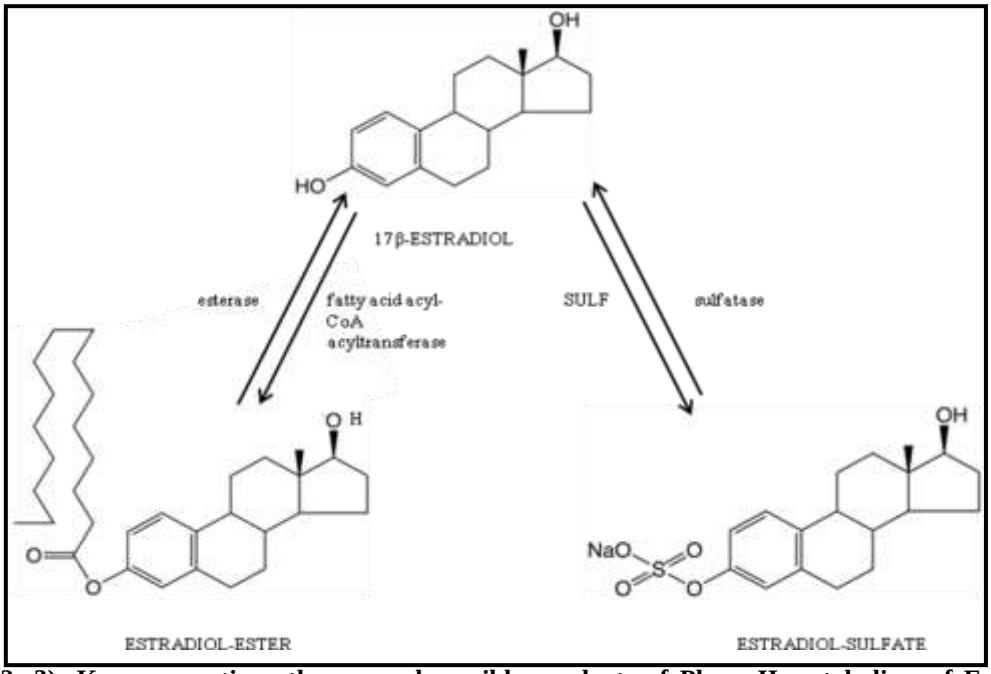


Figure (2- 3): Key enzymatic pathways and possible products of Phase II metabolism of E_2 (Silvia, 2010).

The mode of action of E_2 is still unknown but there was evidence for a receptor-mediated signal transduction (Köhler *et al*., 2007). Nevertheless no classical vertebrate Estrogen Receptor (ER) has been found on the sea urchin genome, where only Estrogen Related Receptor (ERR) are present (Roepke *et al*.,2006)., the existence of specific cytosolic androgen and estrogen binding sites in the echinoid *P. lividus* and the crinoids *Antedon. mediterranea*, *Asterias. rubens* ,has been reported by radioreceptor assay (Köhler *et al*., 2007).

Furthermore studies on the echinoid *S. purpuratus* has showed that exposure effects to specific ER antagonist can be rescued by adding E_2, further suggesting a receptor-mediated signal transduction (Unuma *et al*, 1999).

2-4-Coelomocytes:

Echinoderms are invertebrates and include a number of species with key roles in the marine ecosystem. Many species, living in coastal and estuarine waters, are directly exposed to potentially pathogenic microorganisms and have developed defense responses mainly based on immunocytes and humoral factors contained in the coelomic fluid (Pancer, 2000).

The morpho-functional properties of the immunocytes are still controversial and their immune mechanisms are, in part, unknown. In echinoids, four main coelomocyte types have been described: amoebocytes, vibratile cells, red cells and uncoloured spherulocytes , amoebocytes and spherulocytes represent the main coelomocyte populations that seem to be responsible for a wide repertory of immunological functions, cellular recognition, phagocytosis, cytotoxicity antibacterial activity , inflammatory reactions , including C3-like expression after activation with LPS ,prophenoloxidase activity ,capsule formation and graft reaction (Silva and Peak,2008).

In the sea urchin *Paracentrotus. lividus*, a calcium-dependent cytolytic activity has been attributed to cytoplasmic granules separated through a Percoll density gradient from amoebocyte populations. Moreover, an innate antibacterial activity against *Vibrio. alginolyticus* has been shown in coelomocyte lysates, and in cell-free coelomic fluid (Drago *et al*., 2009).

The coelomic cavity of the sea urchin contains cells, generically called coelomocytes, that have been studied for many decades, due to their capability to respond to injuries, host invasion, and cytotoxic agents, coelomocytes were regarded as the immune effectors of the sea urchin.

In fact, coelomocytes react to challenges with modifications in their motility, increased phagocytic and encapsulation activities, and release of cytotoxic factors (Smith *et al*., 2006).

Coelomocytes can be separated into subpopulations whose functions are not yet understood, It is not yet clear whether diverse coelomocytes, which differ in size and shape, have different functions or whether they have a common precursor cell resembling the human blood cell lineages (Silva, 2000). coelomocyte represent circulating stem cells (CSC), freely moving in the coelomic fluids and produced by the proliferating coelomic epithelium (coelothelium).Apart from their typical roundish shape, they are morphologically comparable to amoebocytes. CSC do not represent reserve stem cells already present in the tissues, but are rather pluripotential elements produced by the continuous turnover of the coelothelium . Since they derive directly from coelothelial proliferation, this implies that the coelothelial cells (peritoneocytes) are plausibly partially involved in either dedifferentiation or Tran's differentiation phenomena (Candia Carnivali, 1997).

Also CSC contribute to all the regeneration process phases, they migrate massively along the coelomic canals towards the amputation site during the overall regeneration period, Their contribution to regenerating tissues seems to be restricted to the coelomic compartments(Xing *et al*.,2008).

In fact, once migrated into the wound area, they form first typical clots which seal the injured coelomic canals and later give rise to the re-growth of the coelomic components, so CSC can be considered the progenitor cells of all the coelom-derived differentiated cells, including peritoneocytes, myocytes, neurons of the plexus and free coelomocytes, although a possible extra-coelomic contribution to tissue re-growth cannot be excluded (Beck, 1996).

2-4 -Coelomocytes functions.

The defense capabilities of echinoderms were defined from allograft rejection experiments, but research has also concentrated on characterizing and understanding the functions of the coelomocytes, the cells that mediate these responses, Coelomocytes are found in the coelomic fluid and depending on the species, can be a mixture of several morphologically different types (Ito *et al*., 1996). Sea urchins have four types of coelomocytes which include phagocytes (present in all echinoderms, also called bladder, petal or amoebocytes), red spherule cells (also called eleocytes, morula cells and pigment cells), colorless spherule cells (also called white morula cells) and vibratile cells (Gross *et al*., 1999a).

2-4-1- Coelomocyte Infiltrates in Injury and Infection.

Coelomocytes have long been considered to be mediators of the immune response, in part ,because of their presence surrounding injuries and infections in several echinoderm species and their appearance in grafted tissues, skin infections become ringed with black or dark red tissues that are accumulations of red spherule cells in the sea urchin, *Paracentrotus lividus* and in S. *purpuratus* , infiltrates of red spherule cells have also been documented as red spots on gonadal tissue of the sea urchin *Strongylocentrotus intermedius*. that surround metacercaria of a parasitic worm(Shimizu *et al*.,1999).

Cellular infiltrates consisting of phagocytes and red spherule cells have also been noted around broken, infected, and regenerating spines of S. *purpuratus* . Red pigmented cells accumulated at sites of surgical injuries during the initial phase of tissue transplantation in *L. pictus* and histological analysis of allografts in the sea star, *D. imbricata* revealed mixed cellular infiltrates in which cell densities increased during the chronic rejection process (Lin *et al*., 2001).

2-4-2 .Coelomocyte Responses to Foreign Substances.

The survival of echinoderms in the microbe-rich marine environment is dependent on their ability to defend themselves against microbial invasion. Combating infections must include rapid, efficient, and sometimes selective clearance of foreign invaders in order to animal survives. The capability of echinoderms to eliminate injected pathogens, foreign cells, and other types of particles has been well documented (Kudriavtsev and Polevshchikov, 2004). Injection of bacteria into the coelomic cavity of the sea star *D. imbricata* or into the sea urchins, *Echinus. esculentus* , *S. purpuratus*, or *Strongylocentrotus droebachiensis* were efficiently cleared in a few hours to a few days(Haug *et al*.,2002).

In contrast to the sterile coelomic fluid of sea urchins, pre-injection analysis of coelomic fluid from the sea cucumber, *Parastichopus californicus,* revealed the presence of a natural bacteria with as many as 108 bacteria per milliliter of coelomic fluid , Consequently, bacteria injected into the coelomic cavity of P. *californicus* were either not cleared or were cleared at varying rates which depended on whether the bacteria were isolated from the gut of the sea cucumber or were obtained from other sources (Matranga *et al*.,2000).

Echinoderms are also capable of clearing xenogeneic cells, foreign non-cellular particles and proteins, injection of cells from the sea urchin, *Arbacia punctulata* into the coelom of the sea star, *Asterias vulgaris* resulted in clumping of the sea star phagocytes, trapping or phagocytosis of the sea urchin cells, and rapid clearance of the injected cells from the coelomic fluid (Beck,1996). The sea cucumber, *Holothuria polii* efficiently phagocytosed and encapsulated injected red blood cells (RBCs) , as did the sea urchin *S. droebachiensis* , the sea urchin, *L. pictus, e.* • *ciently* cleared T4 bacteriophage and carmine particles injected into the coelomic cavity of *P.calsifornicu*

resulted in agglutination, encapsulation and brown body formation followed by the excretion of the brown bodies into the cloaca, even latex beads were quickly cleared from the coelomic cavity (Chia and xing,1996).

In addition to using repeated allografting to assess the specificity of immune memory in echinoderms, repeated injections of foreign substances have also been performed. However, no differences have been noted in clearance rates of bacteria injected multiple times into the coelomic cavity of *S. purpuratus*, regardless of the interval between inoculations. Similarly, accelerated (Stabili *et al*., 1996). Clearance rates were not demonstrated when xenogeneic cells were injected a second time into *A. vulgaris*, nor after repeated injections of T4 bacteriophage into *L.pictus* (Pinsino *et al*., 2007).

The activities and functions of coelomocytes have also been studied *in vitro*, where the coelomic fluid from a number of echinoderms has been shown to be bactericidal, Coelomic fluid from *P. lividus* exhibited higher bactericidal activity *in vitro* when coelomocytes were present than after the cells had been removed(Haug *et al*.,2002).

Similar results were reported for coelomocytes from *E. esculentus*, which were found to be bactericidal against *Pseudomonas sp*. and had a wide range of antibacterial activity against both gram negative and gram positive marine bacteria. Lysates of phagocytes and red spherule cells from *P. lividus* were bactericidal against both *Vibrio .spp*. and *Photobacterium sp,* suggested that lysozymes and echinochrome (the pigment of red spherule cells) produced by these cells might mediate the bactericidal activity, in addition, echinochrome-A from *E. esculentus* had effective bactericidal activity against marine bacteria *in vitro* (Plytycz *et al*., 1993).

2-4-3- Cytotoxicity

The phagocyte appears to be the cell type involved in cytotoxic reactions in mixtures of coelomocytes *in vitro*, when phagocytes from the sea urchin *S. droebachiensis* were co-cultured with phagocytes from either E. *esculentus* or *Strongylocentrotus pallidus*, 90% of the cells were killed, and in allogeneic mixtures of S. droebachiensis phagocytes, 70% of the cells were killed (Reunov *et al*.,2004a)**.** On the other hand, others were able to isolate cytolytic granules from phagocytes of P. *lividus*, suggested that these cells mediated their killing function through the release of this cytolytic material (Gross *et al*., 1999b)**.**

2-4-4- Phagocytosis

A subpopulation of coelomocytes from echinoderms are defined by their amoeboid behavior and their abilities to engulf foreign cells and particles, phagocytes from two sea urchin species, *Strongylocentrotus franciscanus* and *S. purpuratus*, were noted to chemo tax towards marine bacteria, with gram positive bacteria being phagocytosed more readily than gram negative bacteria (Silva and peck,2000).

Human and sheep RBCs were taken up within 30 min *in vitro* by phagocytes from the sea urchin, *Strongylocentrotus nudus*, and RBCs opsonized with coelomic fluid from animals that had been pre-injected with RBCs enhanced the phagocytic rate compared to non- opsonized red cells (Pancer, 2000). An important function of the phagocytic cells is degradation of phagocytosed material. Circulating phagocytes from the sea cucumber, *H. polii*, contain a rich selection of lysosomal enzymes, including acid and alkaline phosphotases, b-glucuronidase, aminopeptidase, acid and alkaline protease, and lipase (Gross *et al*., 1999a).

The presence of lysozyme and acid phosphatase has also been documented in the sea cucumber *P. californic*, In addition, arylsulphatase, a lysosomal hydrolyses known to play a role in inflammatory phenomena by catalyzing the hydrolysis of sulfate bonds, has been biochemically detected in coelomocyte lysate preparations of seven different echinoderms and a cDNA from *S. purpuratus* coelomocytes matched to arylsulfatase by expressed sequence tag (EST) analysis (Reunov *et al*., 2004b).

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MATERIALS AND METHODS

3-1: Equipments and materials that used in experiments.

Equipments	Country
Thermostat	Italy
Ultra-microtome	England
Cooling centrifuge	Euro union
Eppendroff centrifuge	Euro union
Transmission electron microscope	Euro union
Confocal microscope	Italy
Light microscope	Italy

Chemical material	Orgin and company
acetone	Sigma(USA)
ASW(artificial sea water)	Italy
oesstradiol powder	Sigma(USA)
SPAFG fixative	Sigma(USA)
Osmic acid	Sigma(USA)
Cacodylate buffer	Sigma(USA)
Ethanol	Sigma (USA)
Epon 812-Araldite	Sigma(USA)
Uranyl acetate	Sigma(USA)
Lead citrate	Sigma(USA)
Trypan blue	Sigma(USA)
BrdU	Sigma(USA)
FdU	Sigma(USA)
paraformaldehyde	Sigma(USA)
gluteraldehyde	Sigma(USA)
Phosphate buffer	Sigma(USA)
Paraffin wax	Sigma(USA)
Methanol	Sigma(USA)
PBS	Sigma(USA)
NGS	Sigma(USA)
PBT	Sigma(USA)
H2O2	Sigma(USA)
Endogenous peroxidases	Sigma(USA)
Nuclease	Sigma(USA)
Peroxidase-anti-mouse	Sigma(USA)
DAP	Sigma(USA)
Distilled water	Italy
Propylene oxide	Sigma(USA)

3-2-Optimizing Animal Stabling Conditions

Paracentrotus lividus adult specimens were collected in the Protected Marine Area of Bergeggi (44°14'N; 8°26'E), on the Ligurian coast of Italy (Tyrrhenian Sea), at 3-5 meters of depth. 10 animals were collected in

December 2010 and immediately transferred, in cool boxes filled with natural sea water, to the laboratory at the University of Milan-Italy.

After their arrival in the laboratory the animals were randomly distributed in 2 aquaria. The 50 L glass aquaria were filled with artificial sea water (Instant Ocean; salinity about 3%, as in the Mediterranean Sea). The tank is provided with internal circulation system as well as mechanical, chemical and biological filters. Animal conditions as well as all physical and chemical parameters were daily (temperature and salinity) or weekly (pH, KH, NO_2, NO_3) monitored throughout the maintenance period. At the beginning of each week filters were cleaned and 10-20% of the sea water was renewed in each aquarium.

Animals were fed once a week with pellets of an artificial diet specifically prepared for sea urchins (Wenger Manufacturing, Inc.). The aquaria were kept at 16 °C and photo-period was kept with 12/12h of light/dark (Birmeline, 1998; Massey, 2004).

3-3-Cell cultures from coelomic fluid.

The coelomic fluid was collected from sea urchin cutting the peristomial membrane with blade and allowing the fluid dropping in an ice-cooled beaker containing 20mL of ISO-EDTA. The liquid was transferred to 15mL Falcon tubes. The suspension was centrifuged at 15 °C, 600 rpm, for 4 minutes, the supernatant was discarded and the cells resuspended in 2mL of ISO-EDTA. The average number of cells was detected by taking one drop of cell suspension and put it into the Burker chamber. The cell suspension was centrifuged at same parameters, the supernatant was discarded and the pellet re-suspended in 2mL of Leibovitz L-15 culture medium. The liquid suspension was transferred in multi-wells and kept at 15 °C (Odintsova *et al*., 1994).

3-4-Cell cultures from gonads.

Gonad explants from sea urchin were placed in 2mL of calcium magnesium free artificial sea water solution (Ca^{2+}-Mg^{2+} -ASW), and left overnight at 15 °C in petri dish.

Gonads were cut off into small pieces and the Ca^{2+}-Mg^{2+} -ASW solution was substituted with a new one. From the petri dish the suspension was taken out and put in a 15ml Falcon tube. The suspension was centrifuged at 15°C, 150 xg, for 4 minutes, the supernatant was discarded and resuspended in 6mL of Ca^{2+}-Mg^{2+} -ASW. Using the same parameters the cells were resuspended in 2mL of cell culture media. The average number of cells was detected by taking one drop of cell suspension and put it into the Burker chamber.

The cell suspension was transferred in a multi-wells and left at 15°C.

Cells viability was checked daily and the culture media was replaced two times per week (remove gently the old culture media close to the surface to avoid to remove many cells). This protocol has been applied with three different types of culture media: MEM, M199 and L-15(Buchanan, 2001).

3-5-Preparation of Oestradiol solutions

Four (4)µg of oestradiol powder were weighed and put it in 10mL of acetone: this solution was named solution (A). From solution (A) 5µL were picked up and put it in 10mL of acetone: this solution was named solution (B). 1µL from solution (B) was taken and put it in 10ml of culture media L-15 to obtain the final value of 20pg/mL of hormone concentration. To get the hormone concentration at 200pg/mL, 10 µL from solution (B) were added to 10mL of culture media. To get the hormone concentration at 2000pg/mL, 100µl from solution (B) were added 10mL of cultures media.

3-6-Oestradiol administration on coelomocytes cell cultures

The cell suspension was prepared according to the protocols previously mentioned and subdivided as follows:

a) Falcon with 2mL of L-15 culture medium with E_2 20pg/mL

b) Falcon with 2mL of L-15 culture medium with E_2 200pg/mL

c) Falcon with 2mL of L-15 culture medium with E_2 2000pg/mL.

The average number of cells was determined by taking one drop of cell suspension and put it into the Burker chamber.

The cell suspension was transferred in a multi-wells and left at 15°C.

Cells viability was checked daily and the culture media was replaced two times per week (remove gently the old culture media close to the surface to avoid to remove many cells)(Barbaglio *et al*.,2006).

3-7-Resin fixation and embedding for LM and TEM

Pellets of cells were fixed with 2% glutearaldehyde in 0.1 cacodylate buffer (PH 7.2) for 1hr and with SPAFG fixative. Samples were washed overnight in the same buffer and post-fixed with 1% osmic acid in 0.1M cacodylate buffer (PH 7.2) for 2hr. After standard dehydration in ethanol series, the samples were embedded in EPON 812-ARALDITE. Ultra thin (50 nm) sections (Reichert ultracut E with diamond knives) were mounted on copper grids and stained with uranyl acetate and lead citrate for electron microscopy, then observed and photographed in Jeol 100SX (Massey,2004).

3-7-Trypan blue exclusion test for cell viability

The dye exclusion test was used to determine the cell number of viable cell present in cell suspension; it is based on the principle that the live cells possess intact cell membranes that exclude certain dyes, such as trypan blue whereas dead cells do not. In this test a cell suspension is simply mixed with dye (1part of 0.4% trypan blue and 1part cell suspension) and then visually examined into the Burker chamber to determine whether cells take up or exclude dye (Scotti,2002).

3-8-Cytochemistry (BrdU detection method)

Cell proliferation was monitored using *in vitro* incorporation of the substituted nucleotide, 5-bromo-deoxyuridine [BrdU], then revealed by a monoclonal antibody against BrdU.

Cell was exposed for 24 hours to culture media in which BrdU and FdU [10:1] were dissolved in a final concentration of 10 µg/mL.

Cells were centrifuged and fixed in paraformaldehyde 4%-glutaraldehyde 0.5% in 0.1M phosphate buffer saline (PBS, pH 7.6) for 2 hours. Following an overnight washing in the same buffer, the samples were dehydrated in a graded ethanol series and embedded in Epon-Araldite 812.

The fixation and embedding protocol maintain good cell integrity providing also good preservation of intigenicity. Semithin sagittal sections were cut and stained as previously described (for providing control sections) or cut and processed for immunocytochemistry. For use with semithin Epon-Araldite sections, the standard BrdU-immunocytochemistry protocol for paraffin wax section was modified as follows.

After a brief treatment (2 min) with a resin-remover mixture (10mL,5mL propylene oxide and 2g NaOH), the sections were washed with methanol (2 min), then wash with PBS (5 min) and incubated (1 hr) with NGS 2% in PBT (Tween 0.05% in PBS) at room temperature, in order to cover a specific sites. A pre-treatment with 0.3% H_2O_2 in PBS was performed to exclude the potential activity of endogenous peroxidases. After several washing with PBS (5 min) the sections were incubated overnight at 4c with anti-BrdU antibody diluted 1:100 in the nuclease.

After several washing in PBS (5 min) the specimens were incubated (3hr) at room temperature with peroxidase-anti-mouse IgG diluted 15:1000 in the diluent ,after a further series of washing in PBS, incubated (5 min) with 0.25%(w/v) 3,3 –diaminobenzidine [DAB] in PBS, and then washed in distilled water. Control reactions were carried out by omitting the primary antiserum and omitting the BrdU-FdU incubation.

Sections were observed under a JENAVAL light microscope provided with a canon S40 coulour camera and canon imaging software (Ganassin *et al*., 2000).

3-10-Coelomocyte Populations Separated Through Discontinuous Density Gradient:

An Iodixinol (Optiprep; Nycomed Oslo, Norway) discontinuous density-gradient was performed as follows: 10, 20 and 30% (v/v) Iodixinol stock solutions in 0.5 M NaCl containing 10 mM EDTA was layered into a 15 mL centrifuge tube. Then, 4 mL of washed coelomocytes adjusted at 1.5×106 mL− 1were layered on the top of the gradient, and the gradient was centrifuged in a Swing-out rotor (800 ×g for 30 min at 7 °C). The resulting cell populations on the top of 10, 20 and 30% Iodixinol were referred to as bands B1, B2, B3 respectively, whereas B4 contained the coelomocytes collected at the bottom. To calculate the percentage of each coelomocyte type, the cell bands were gently removed, washed two times with ISO–EDTA and identified according to (Smith, 1999). Finally, the separated fractions were suspended to obtain an appropriate cell number and used for subsequent assays.

3-10- Light microscopy:

For histology protocols resin embedding/sectioning were employed.
Resin provided high quality histological samples, in terms of tissue preservation. Semithin (0.96μm) sections (Reichert Ultracut E with glass knives) were stained with crystal violet-basic fuchsin and observed under a JENAVAL light microscope provided with a Canon S40 colour camera and Canon imaging software(Willoughby and pomponi,2000).

3-11- Transmission electron microscope (TEM):

Pellets of cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 hr and with SPAFG fixative (for details see Ermak and Eakin, 1976). Samples were washed buffer, and postfixed with 1% osmic acid in 0.1 M cacodylate buffer (pH 7.2) for 2 hr. After standard dehydration in an ethanol series, the samples were embedded in Epon 812-Araldite. Ultrathin (0.07μm) sections (Reichert Ultracut E with diamond knives) were mounted on copper grids and stained with uranyl acetate and lead citrate for electron microscopy, then observed and photographed in a Jeol 100SX electron microscope (Massey, 2004).

3-14-Cultur Media Components:

3-14-A-Leibovitiz **Medium(L-15 medium)**

Inorganic Salts	**mg/l**
$CaCl_2 \cdot 2H_2O$	185.00
KCl	400.00
KH_2PO_4	60.00
$MgCl_2 \cdot 6H_2O$	200.00
$MgSO_4 \cdot 7H_2O$	200.00
NaCl	8000.00
$Na2HPO_4$ (anhyd.)	190.00
Amino Acids	**mg/l**
L-Alanine	225.00
L-Arginine	500.00
L-Asparagine	225.00
L-Cysteine	120.00
L-Isoleucine	250.00
L-Leucine	125.00
L-Lysine	75.00
L-Methionine	75.00
L-Phenylalanine	200.00
L-Serine	200.00
L-Threonine	300.00
L-Tryptophan	20.00
LTyrosine	300.00
DL-Valine	100.00
Vitamins	**mg/l**
D-Ca Pantothenate	1.00
Choline Chloride	1.00
Folic Acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxine•HCl	1.00
Flavin Mononucleotide	0.10
Thiamine•Monophosphate HCl	1.00
Other Components	**mg/l**
D(+) Galactose	900.00
Phenol Red•Na	10.00
Sodium Pyruvate	550.00
Glycine	250.00

In order to obtain an isomic solution for marine invertebrate: Solution enrichment for Leibovit's l-15 medium and related addition of salts was provided.

Salts	g/l
NaCl	20.2
Kcl	0.54
$CaCl_2$	0.06
$NaSo_4$	1.00
$MgCl_2$	3.09
Ribonucleosides	mg/l
Adenosine	10
Cytidine	10
Guanosine	10
Uridine	10
Deoxyriboncleosides	mg/l
2 Deoxy adenosine	10
2 Deoxy cytidine	10
2 Deoxy guanosine	10
2 Deoxy thymidine	10

500 mL flasks were prepared as above mentioned, ultra-filtered and subdivided in 50 mL sterile bottles. Modified Leibovit's was stored at 4°C before using.

The Following components were also added:

Gentamicin	40 ul /50 ml
Insulin	250 ul / 50 ml
FCS	1 ml /50 ml
Glutamine	500 ul /50 ml
Glucose	1 mg /ml

3-14-B- Medium 199(M 199)

Inorganic salt	**mg/l**
Calcium chloride	20
Ferric nitrate	0.072
Magnesium sulfate	9.767
Sodium acetate	5.0
Sodium chloride	6.8
Sodium phosphate	12.2
AMINO ACIDS	mg/l
L-Alanine	250
L-Arginine	700
L-Aspartic Acid	600
L-Cystine	1.1
L-Glutamic Acid	260
Glycine	668
L-Histidine	2.1
Hydroxy-L-Proline	1
L-Isoleucine	2.3
L-Leucine	6
L-Lysine	7
L-Methionine	1.5
L-Phenylalanine	2.5
L-Proline	4
L-Serine	3
L-Threonine	3
L-Trptophan	1
L-Tyrosine	5.766
L-Valine	5
VITAMINS	mg/l
Ascorbic Acid	0.0056
D-Biotin	0.001
Calciferol	0.1
Choline chloride	0.05
Folic Acid	0.001
Menadione	0.0061
Myo-Inositol	0.005
Niacinamide	0.0025

Nicotinic Acid	0.0025
Retinol acetate	0.0014
Riboflavin	0.001
Thiamine	0.001
OTHER	mg/l
Adenine Sulfate	1
Adenosine triphosphate	0.1
Adenosine monophosphate	0.0238
Cholesterol	0.02
Deoxyribose	0.05
Glucose	100
Guanine	0.03
Hypoxanthine	0.03
Xanthine	0.0344
Adenine Sulfate	1
Adenosine triphosphate	0.1
Adenosine monophosphate	0.0238
Cholesterol	0.02
Deoxyribose	0.05
Glucose	100
Guanine	0.03
Hypoxanthine	0.03
ADD	mg/l
Sodium Biocarbonate	220

3-14-C-Minimum Essential Medium Eagle (MEM)

Inorganic Salt	mg/l
Calcium Chloride	20
Magnesium Sulfate	9.767
Potassium Chloride	40
Sodium Chloride	680
Sodium Phosphate Monobasic	12.2
AMINO ACIDS	mg/l
L-Arginine.HCL	126
L-Cystine.2HCL	3.13
L-Glutamine	2.92

L-Histidine.HCL.	2.4
L-Isoleucine	5.2
L-Leucine	5.2
L-Lysine	7.725
L-Methionine	1.5
L-Phenylalanine	3.2
L-Threonine	4.8
L-Tryptophan	1.0
L-Tyrosine	5.19
L-Valine	4.6
VITAMINES	mg/l
Choline Chloride	0.1
Folic Acid	0.1
Myo-Inositol	0.2
Niacinamide	0.1
Hemicalcium	0.1
Pyridoxal.HCL	0.1
Riboflavine	0.01
Thiamine.HCL	0.1
OTHER	mg/l
Glucose	100
Phenol Red.Na	1.1
ADD	mg/l
Sodium Bicarbonate	220

3-15-Statistical analysis:

The statistical analysis system –SAS (2004) program was used to the effect of factors (culture media & period) in cell viability. The chi-square test was used to the significant compare between percentages in this study.

4-1: Effect of culture media on coelomocytes viability.

cell viability of the coelomocytes from *p.lividus* survived but did not grow, in each of the three culture media tested .three days after incubation, the cell viability was 95 % in L-15, 85% in MEM and 80 % in M199 while 10%in control group. The statistical analysis of the results reveals that there is significant difference ($P<0.01$) between treated groups with cultures media

and control group. However, over the next three days the viability of the coelomocytes in L-15, MEM and M199 decrease to (85, 30, 25) % respectively.

Figure (4-1) illustrated that there is significant differences ($P<0.01$) in the viability of coelomocytes in the treated groups with culture media after 6 days of incubation compared with control group.

By day 9, the coelomocytes viability was 60% in L-15 but only 10% in MEM and 5% in M199 the statistical analysis showed that significant difference ($P<0.01$) was observed in the treated group with cultures media compared with control group.

By day 12, the coelomcytes viability was 40% in L-15 but fall dramatically to 0% in rest cultures media. The results showed a significant decrease ($P<0.01$) in the viability of coelomocytes in the treated group with cultures media compared with control group with the same period of incubation.

The coelomocytes still alive in L-15 at 15 days with viability (15%) while all coelomocytes had died in MEM, and M199. There is significant decrease ($P<0.05$) in treated groups with cultures media as compared with the control.

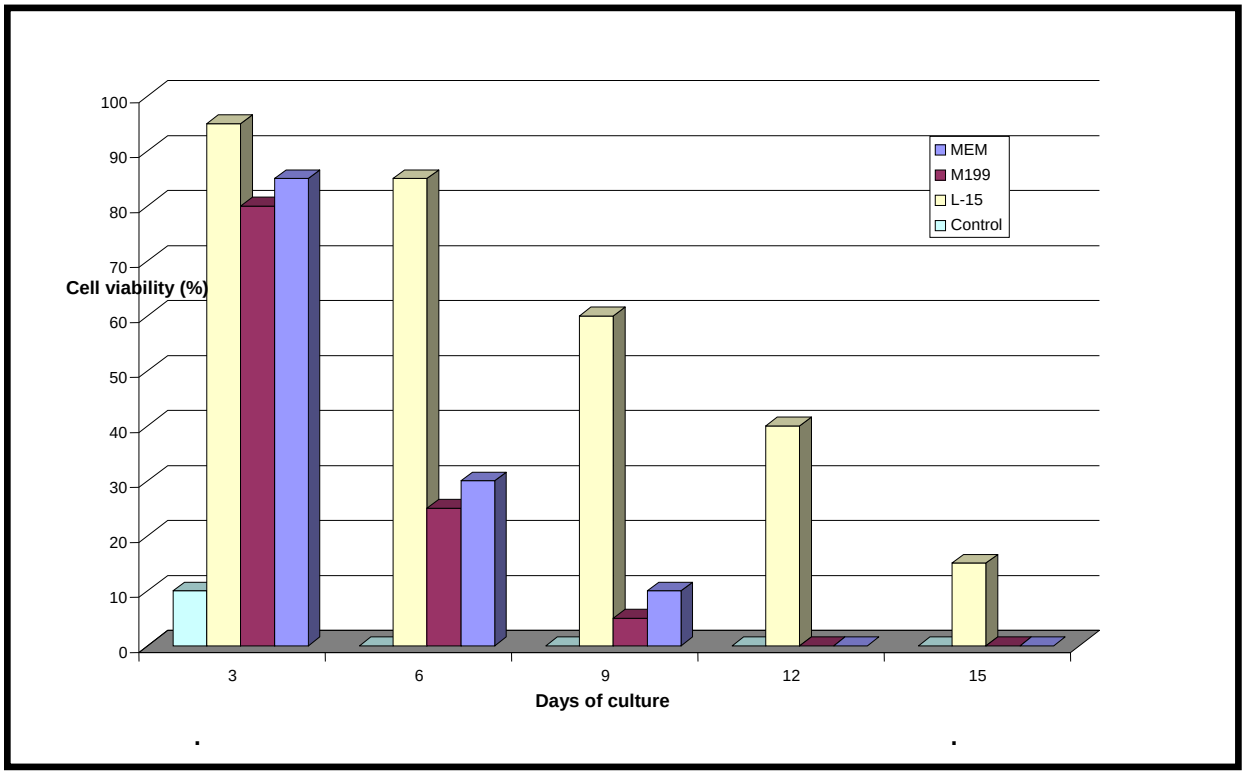


Figure (4-1): the viability of coelomocytes primary cell culture for sea urchin (*p.lividus*), using different culture media

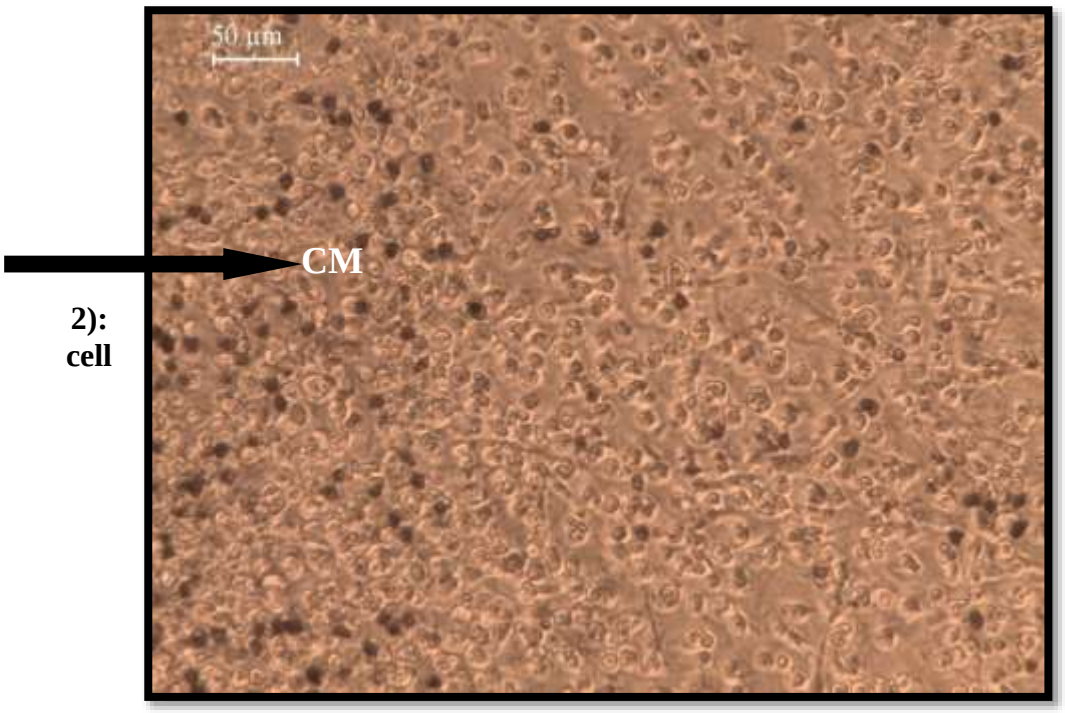


Figure (4-2): primary cell culture of coelomocytes for sea urchin (*p.livdus*), using L-15 medium. After 6 days incubation with culture media (scale bar=50µm, L.M) CM: Coelomocytes

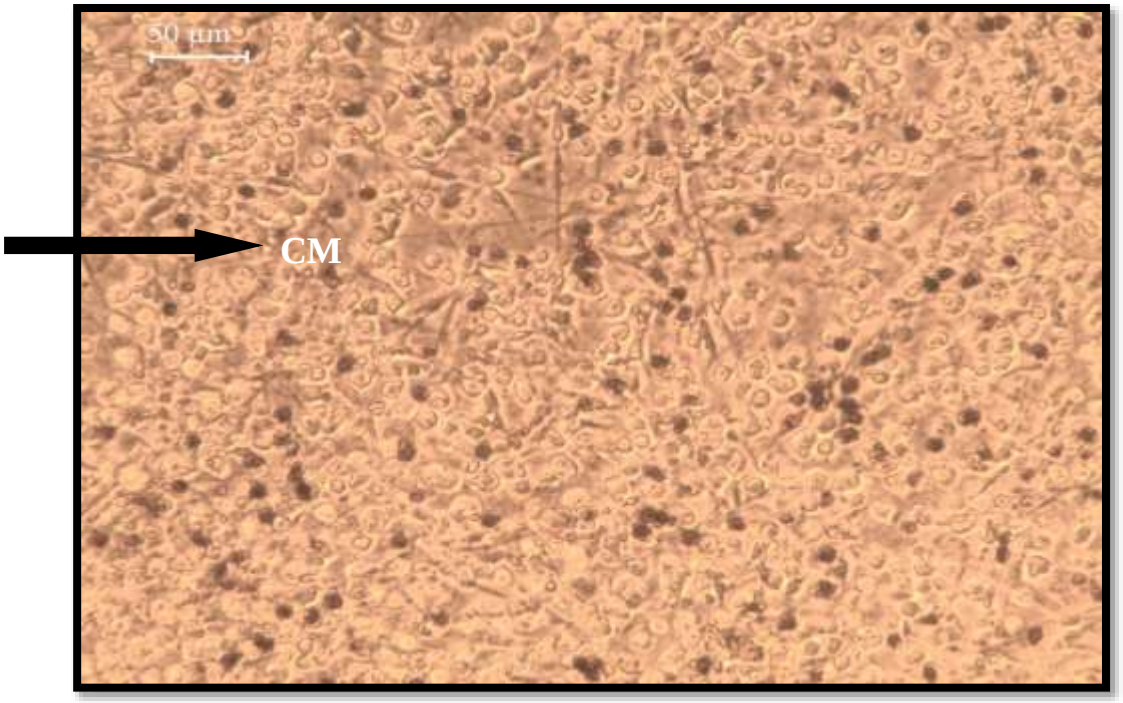


Figure (4-3)

Primary cell culture of coelomocytes for sea urchin (*p.lividus*), using MEM medium after 6 days of incubation with culture media(scale bar=50µm, L.M) CM: Coelomocytes

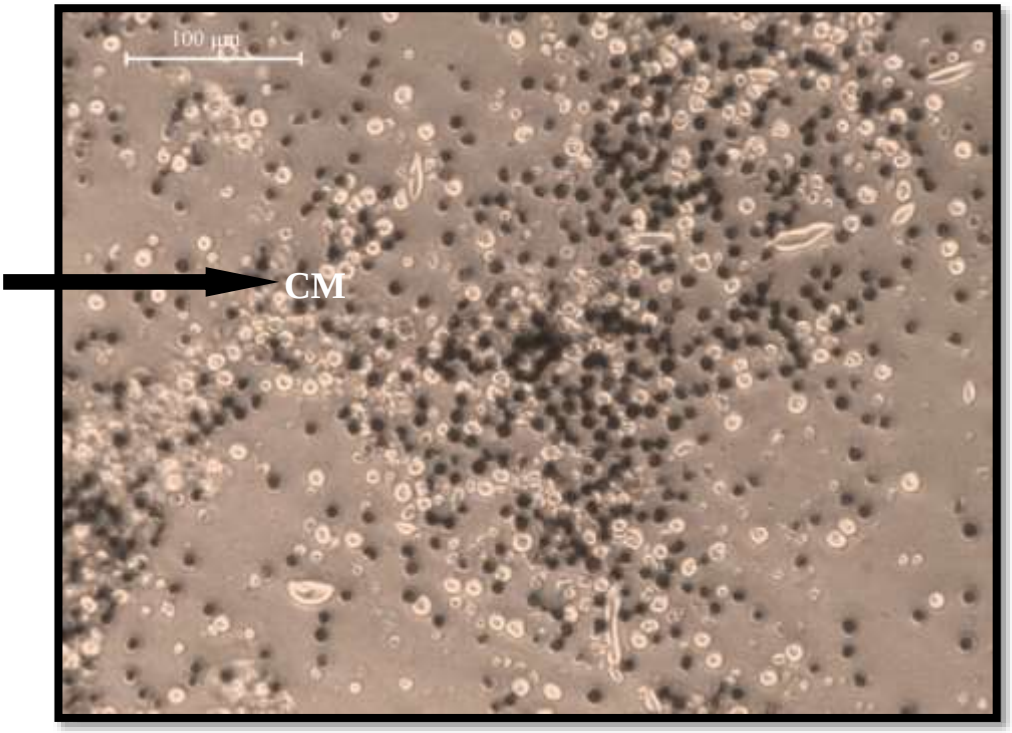


Figure (4-4) Primary cell culture of coelomocytes for sea urchin(*p.lividus*), using M199 medium after 6 days of incubation with culture media(scale bar=50µm, L.M) CM: Coelomocytes

4-2- Effect of culture media on the gonad cells viability.

The results indicated that significant difference ($P<0.01$) in viability of gonads cell in treated groups with culture media compared with control group. After 3 days, gonads cultured in L-15, MEM and M199 maintained with high viability (90, 80, and 75) % respectively. However over the next three days the viability of cells culture in MEM, M199 decreased to 35% and 30% respectively which were lower than the viability of cells in L-15 medium 80% (figure 4-5).

Also, the results showed a significant difference ($P<0.01$) of treated group with cultures media compared with control group after 6 days of incubation (figure 4-5).by day 9 the viability was 70% in L-15 medium, whereas the viability decreased in MEM, M199 to 15% and 10% respectively, and the results showed significant difference ($P<0.01$) in treated group with cultures media compared with control group after 9 days of incubation.

By 12 day the viability of gonad cell was 30% in L-15 medium while the all cells in MEM,M199 media had died and the results showed significant difference($P<0.01$) in treated group with cultures media compared with control group after 12 days of incubation. By 15 day the gonad cells still a live with low viability (10%). there is significant decrease to ($P<0.05$) in treated groups with cultures media compared with control group.

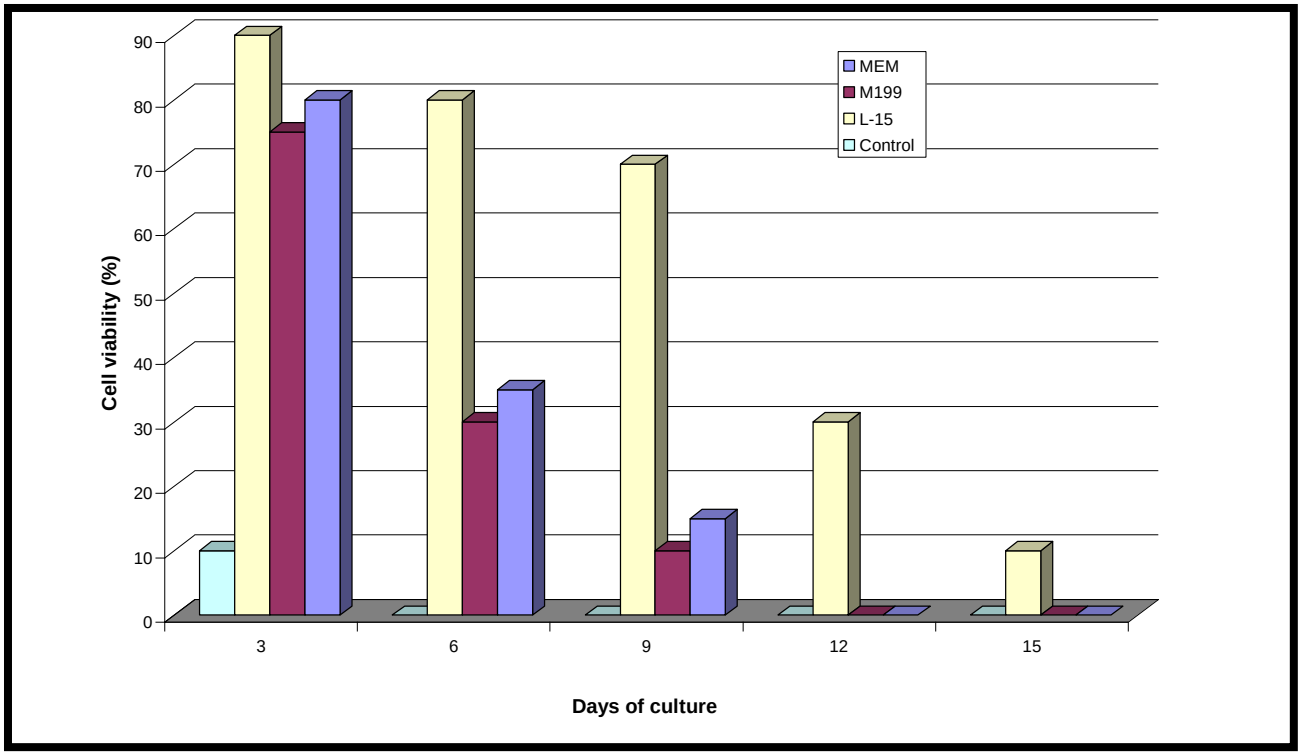


Figure (4-5): Primary cell culture of gonad cells for sea urchin (*p.lividus*), using different culture media

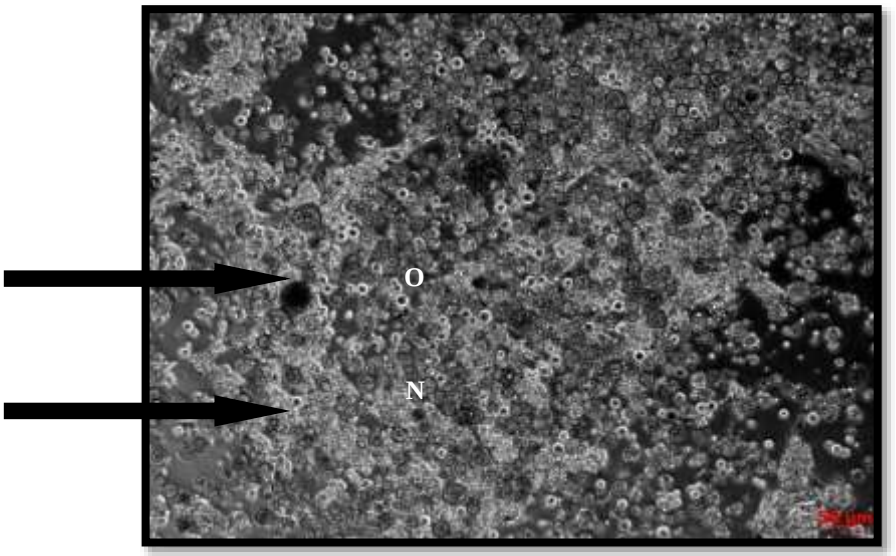


Figure (4-6). Primary gonad cells culture of sea urchin (*p.lividus*), usingL-15 medium after 6 days of incubation (scale bar=50µm, TEM). O:Oocytes, N: Nutrient Phagocytes

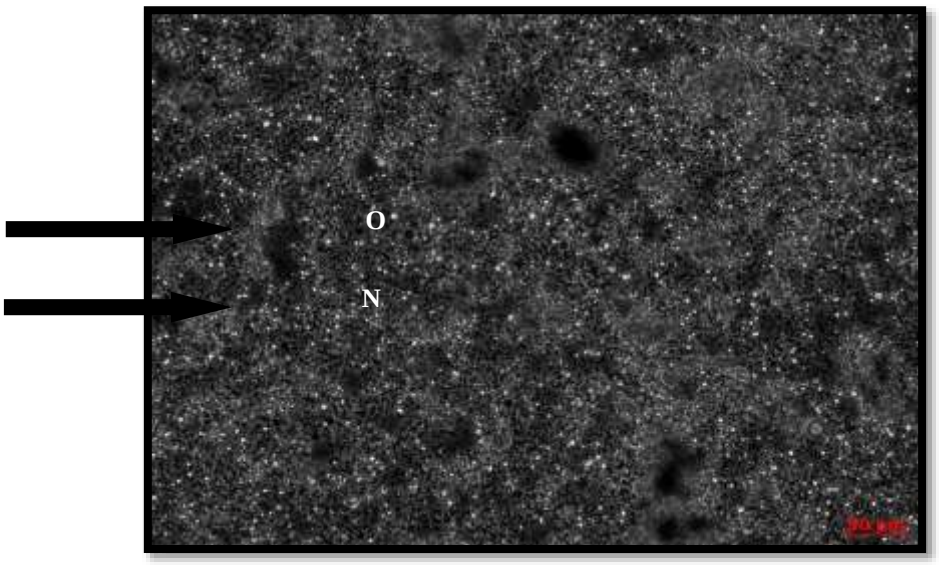


Figure (4-7).primary gonad cells culture of sea urchin (*p.lividus*), using M199 medium after 6 days of incubation, cell appear small and irregular shape (scale bar=50µm, TEM). O:Oocytes, N: Nutrient Phagocytes

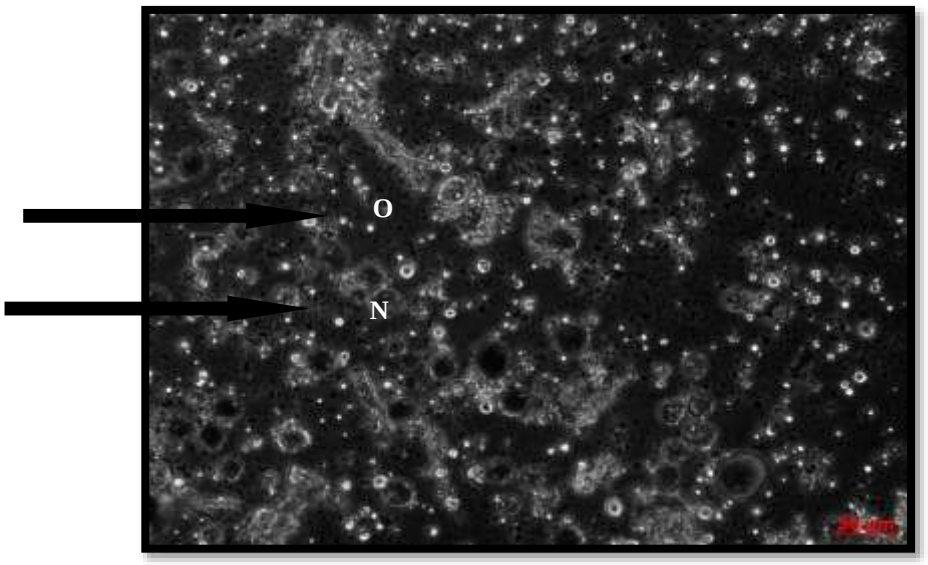


Figure (4-8). Primary gonad cells culture of sea urchin, using MEM medium after 6 days of incubation, cells appear small and irregular shape(scale bar=50µm, TEM).O:Oocytes, N: Nutrient Phagocytes

4-3- Effect of culture media on the viability of coelomocytes and gonad cells.

Three different culture media L-15, MEM and M199 were used individually as basal media to develop appropriate media for good cell yield and cell adherence.

L-15 media was tested for its quality in promoting cell viability and cell growth by various authors, Wen *et al*. (1993a, b) found that L-15 medium was a suitable basic medium for *in vitro* cell cultures of oyster and hard clam tissue and the performance of individual basal medium has limited potential in cell yield.

Cornet (2006) investigated the growth promoting activity of the serum used for medium supplementation in the primary mantle tissue culture from the bivalve mollusc M. *galloprovincialis* and reported that best results were obtained using 20% chicken serum and 30% fetal calf serum with L-15 medium that similar to the present study, the performance of the basal medium L-15 was better than MEM and M199 in cell viability

Naganuma *et al*. (1996) maintained the culture of cells of trochophore larvae of the abalone, *H. discus* for 20 days in a modified L-15 Medium,this media formulated by Ellis and Bishop (1998) containing various tissues extracts promoted the growth of oyster cells up to 42 days. In the present study, the cultures were maintained to a maximum of 15 days.

Carefoot *et al*. (2000) studied the viability of the isolated cells of the abalone, *Haliotis kamatschatkana* and confirmed that the cells exhibited less than 5% staining with Trypan blue and actively synthesized glycogen following the addition of glucose substrate and amino acids to the culture medium.

Sud *et al*. (2001) used water-soluble matrix (WSM) fraction, extracted from the nacre of *Pinctada maxima*, in the regulation of cell activity in abalone mantle cell culture (*H. tuberculata*). The extract of WSM reduced the global viability of mantle cells.

In the present study the coelomocyte cell culture was maintained up to 15days that indicated the suitability of the L-15 with supplements for the maintenance of explants cell culture for *p.lividus*.

In order to maintain cell growth, medium containing sufficient growth factors and nutrients is needed. L-15 medium contains asparagines or arginine which is present at high concentrations in sea urchin body fluid or in soft tissue extract. Therefore, addition of these substances to the growth medium was considered necessary to enhance the survival of coelomocytes and the gonads cell. In addition, the use of fetal bovine serum (FBS) at concentrations of 10–20% has been reported to be beneficial for bivalve tissue cultures (Birmelin *et al*., 1999; Cao *et al*., 2003).

When fetal bovine serum (FBS) has been used as a supplement to basal growth medium, most of the time it was heat-inactivated before use in tissue culture. For example, this was reported for shrimp tissue culture (Mulford *et al*., 2001; Lang *et al*., 2002).

This procedure was intended to destroy complement components in the serum and therefore prevent cells from being lysed during antibody binding (Leshem *et al*., 1999). Since it has been demonstrated that functionally and structurally similar components to those of the mammal complement are present in all non mammalian vertebrates such as birds (Cao *et al*., 2003).

To obtain a superior growth medium for the support of Pacific oyster cell growth *in vitro*, a medium containing hormones, growth factor, antioxidants and lipids which may enhance cell viability has been reported (Zang *et al*., 2003).

In agreement with previous studies, the efficiency of Leibovitz's L-15 medium for promoting growth and cell survival of invertebrate tissues was

demonstrated. In fact, L-15 medium was the most effective for cell growth and survival whereas with MEM and M199 media, the results were generally poor. In particular, MEM and M199 media revealed pH problems: they showed variable pH shift that could influence cell conditions. In fact the best pH for growth of marine invertebrate cells is greater than 7.0. Indeed, pH values of 7.0–7.4 were reported for several species of crustaceans. (Barcia *et al*., 1999)

The sustainability of L-15 medium with gonad extract was evidenced by subcultures. Chen and Wen, (1999).used a medium containing hormones, growth factor antioxidants and lipids with an aim to study viability of cells of pacific oyster, *C. gigas* and to formulate a superior growth medium.

Waltone and Smith (1999) used the extract from oyster reproductive gland and found to prolong the survival of oyster cells *in-vitro*. The results are identical to the present study where the L-15 basal medium with 10% GE, ME and WBE gave slightly better cell yield.

The cell will release organic osmolytes, especially free amino acids and their derivatives, through its membrane, these compounds have been termed "compatible solutes" because of their compatibility with cellular metabolism .This excretion would be possible owing to changes of membrane permeability, related to phosphorylation of some specific proteins, themselves Ca2+-dependent (Perrino and Pierce, 2000a).

Authors identified only a limited number of osmolytes involved, primarily amino acids (Alanine, Isoleucin , Arginine) and two amino acid derivatives, glycine betaine and taurine betaine. It is likely that, has significant implications for metabolic and functional integrity of mantle cells that are directly involved in the regulation of the cell division cycle.
(Martin *et al*., 1999).

The amino acids , in particular those which are contained in L-15 such as Threonine and lysine which have been reported in mantle tissues of *M.*

galloprovincialis, would be involved in bivalves for cellular volume regulation. In fact, it is possible that all the free amino acids behave as osmolytes which allow the control of cellular integrity and directly involved in the regulation of cell division cycle (Deaton, 2001).

Our results showed that the high concentrations of amino acids may prolong the survival of the coelomocytes and gonad cells of *P.lividus in vitro*. These results are similar to those obtained by others previous studies demonstrated that L-15 medium is a suitable basic medium for the *in vitro* cell culture of oyster and hard clam tissue. It was also reported that explanted culture of heart tissue cell of the oyster could be sub cultivated 6 times by using L-15 medium with oyster gonad extracts and rabbit or bovine pituitary gland extracts (Muller *et al*., 2000).

Experiments performed on hard clam showed that medium containing pronase or collagenase may promote cell proliferation (Ganassin *et al*., 2000). Using the present culture system, high viability of coelomocytes and gonads cell was observed.

A variety of culture media have been tested for the primary culture of crustacean tissues, these include Leibovitz L-15, PRMI 1640, Medium 199, and several specifically formulated media (Waltonand Smith, 1999).

Leibovitz L-15 has been considered the best commercial medium for tissue culture of crustaceans (Sheedan, 2000).

In our study, Leibovitz L-15 medium supported cell survival in the first 6 days of culture , however, the Modified L-15 medium yielded better results for longer term viability (by day 15). The higher concentration of glutamine and glucose in L-15 medium apparently benefited the survival of *in vitro* cultured coelomocytes and gonad cells of the oyster

Fetal bovine serum (FBS) is often used as a supplement in crustacean and mollusk cell culture (Sano, 1998; Chen and Wang, 1999; Walton and

Smith, 1999). In this study, fetal bovine serum (FBS) enhances the viability in whole the coelomocytes and gonads cell cultures even at a low concentration of 5%. FBS presumably have been used to support cell survival or may be have potential role to induce coelomocytes and gonads cell viability.

superior growth and sustained culture viability could be achieved by amplifying the basal levels of amino acids and vitamins, High density coelomocytes growth was achieved in developing the L-15 media series by elevating various nutrients while maintaining relatively constant salt content and medium osmolality, successful results have been obtained with a simple fold-amplification of amino acids (and other nutrients) within the basal formulation (Bibila *et al*., 1994; Mahadevan *et al*., 1994).

The presence of essential nutrients and the absence of inhibitory compounds may be critical to determine the short term survival; other factors of the cell culture environment can have a profound effect on long term viability in culture. In particular, cell-to-substrate attachments have been demonstrated to be important for promoting the metabolism and long term viability of many vertebrate and marine invertebrate cells (Odintsova *et al*., 2000, Shimizu *et al*., 2001).).

We thus selected L-15 and modified it by addition of mineral salts in order to raise its osmolarity , adjust its Na/K ratio and its Ca^{2+} and Mg^{2+} concentrations.

Other workers hypothesized that the high concentrations of amino-acids and galactose of this medium, it made the assumption that the culture's viability might not be limited by the consumption of substrates provided by the medium, but rather by the absence of growth-promoting substances, NCS and FBS may be also considered key factors for promoting coelomocytes and gonads cell (Moss *et al*., 1998).

4-4- The effect of Oestradiol (E_2) on the viability of coelomocytes.

The results showed a significant difference ($P<0.01$) in the viability of coelomocytes in treated group with cultures media and (20, pg /ml) of hormone compared with control groups. The cells were still alive at day 15 in L-15 medium, while the cells were still alive at day 9 in MEM and M199 media (figure 4-9, 4-10, and 4-11).

Also the results indicated that a significant difference ($P<0.01$) in the viability of coelomocytes in treated group with cultures media and (200pg/ml) of hormone compared with control groups.

The cells were still alive at day 15 in L-15 medium while the cells were still alive at day 9 in MEM and M199 media.

The statistical analysis of the results reveals that there is significant difference ($P<0.01$) **in the viability of coelomocytes in treated group with cultures media and (2000pg/ml) of hormone compared with control groups. the cells were still alive at day 18 in L-15 (figure 4-9).while the cells were still alive at day 12 in MEM and M199 media (figure 4-10,4-11).**

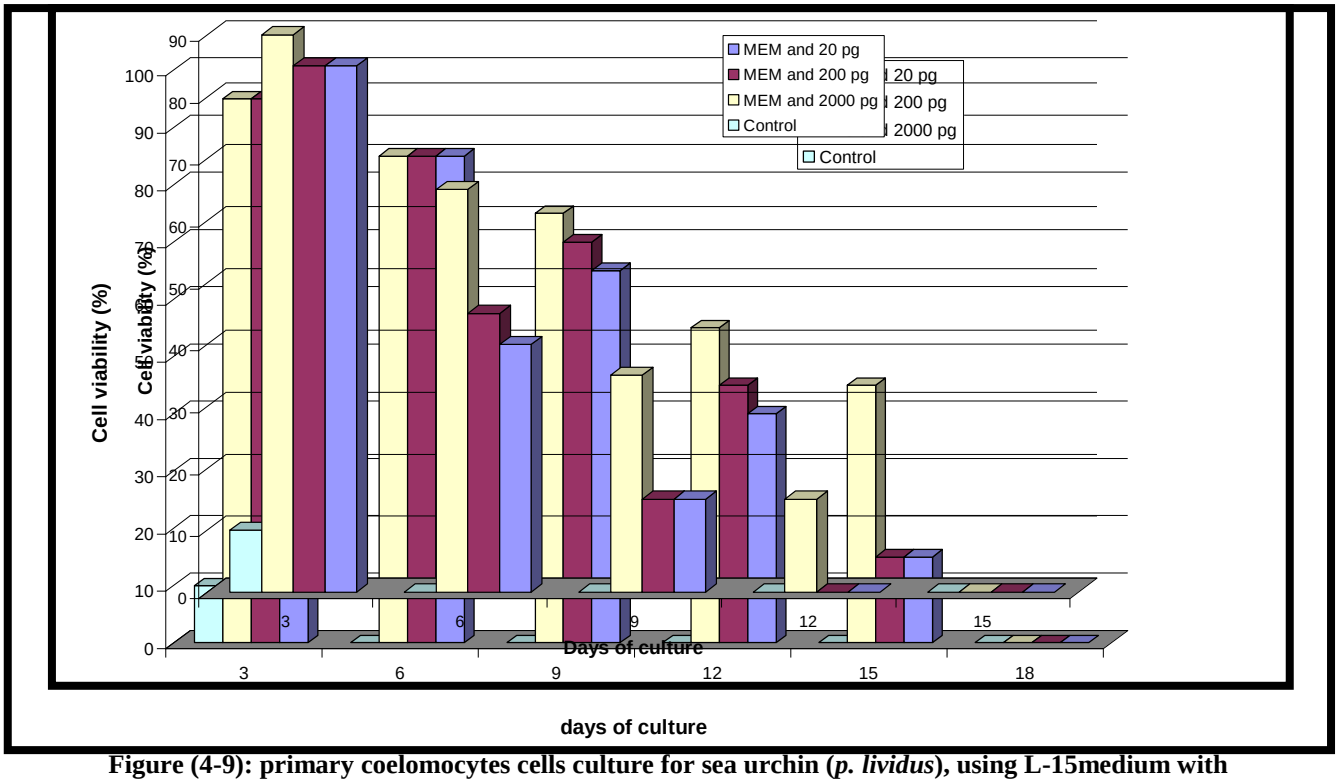


Figure (4-9): primary coelomocytes cells culture for sea urchin (*p. lividus*), using L-15medium with different concentration of oestradiol (E_2).

Figure (4-9) illustrated that there was significant differences (P<0.01) in the viability of coelomocytes in L-15 medium combined with 20pg/mL concentration of E_2 compared with control groups, and there was significant differences (P<0.01) in the viability of coelomocytes in L-15 medium combined with 200pg/mL concentration of E_2 compared with the control groups.

The figure also showed that significant differences (P<0.01) in the viability of coelomocytes in L-15 medium combined with 2000pg/mL concentration of E_2 compared with control groups.

Figure (4-10): Primary coelomocytes cells culture of sea urchin(*p.lividus*), using MEM medium with different concentration of E2.

Figure (4-10) showed that there was significant differences ($P<0.01$) in the viability of coelomocytes in MEM medium treated with 20pg/mL concentration of E_2 compared with control groups, there was also significant differences($P<0.01$) in the viability of coelomocytes in MEM medium treated with 200pg/mL concentration of E_2 compared with control groups.

The figure also showed that significant differences ($P<0.01$) in the viability of coelomocytes in MEM medium treated with 2000pg/mL concentration of E_2 compared with control groups.

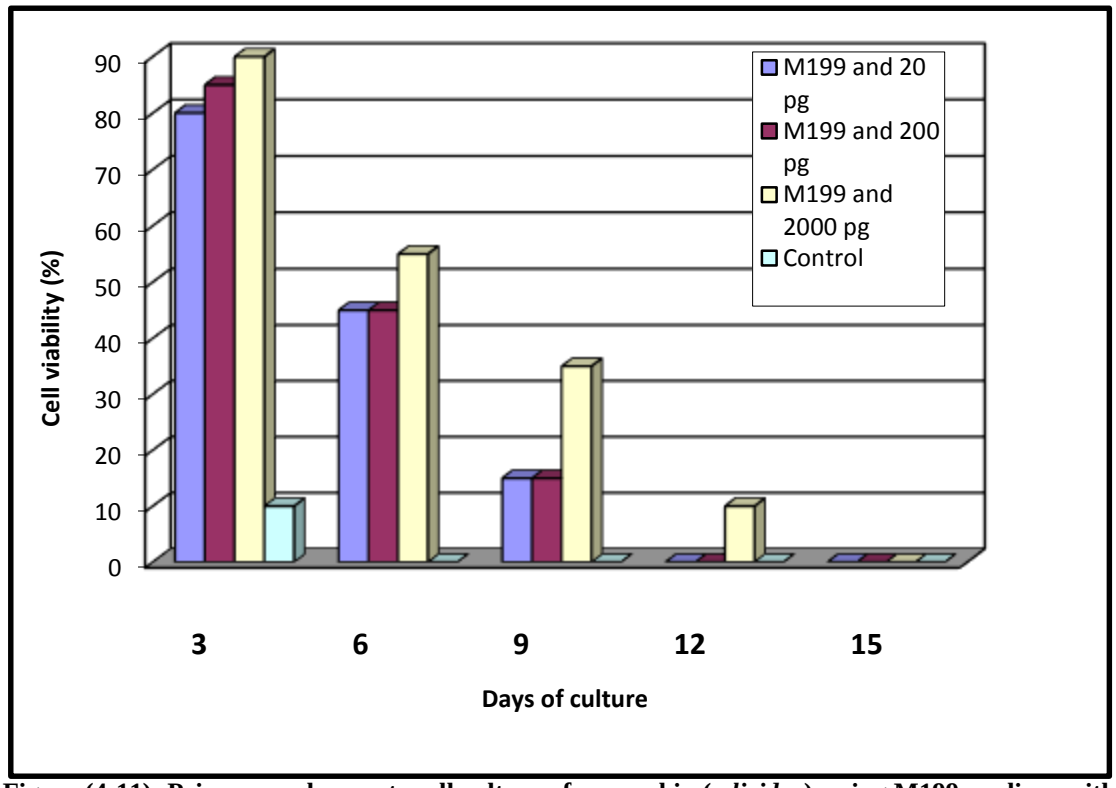


Figure (4-11): Primary coelomocyte cell culture of sea urchin (*p.lividus*), using M199 medium with different concentrations of E_2.

Figure(4-11)showed that significant differences ($P<0.01$) in the viability of coelomocytes in M199 medium treated with 20pg/Ml concentration of E_2 compared with control groups, also there was significant differences in the viability of coelomocytes in M199 medium treated with 200pg/mL concentration of E_2 compared with control groups.

The figure also showed that significant differences ($P<0.01$) in the viability of coelomocytes in M199 medium treated with 2000pg/mL concentration of E_2 compared with control groups.

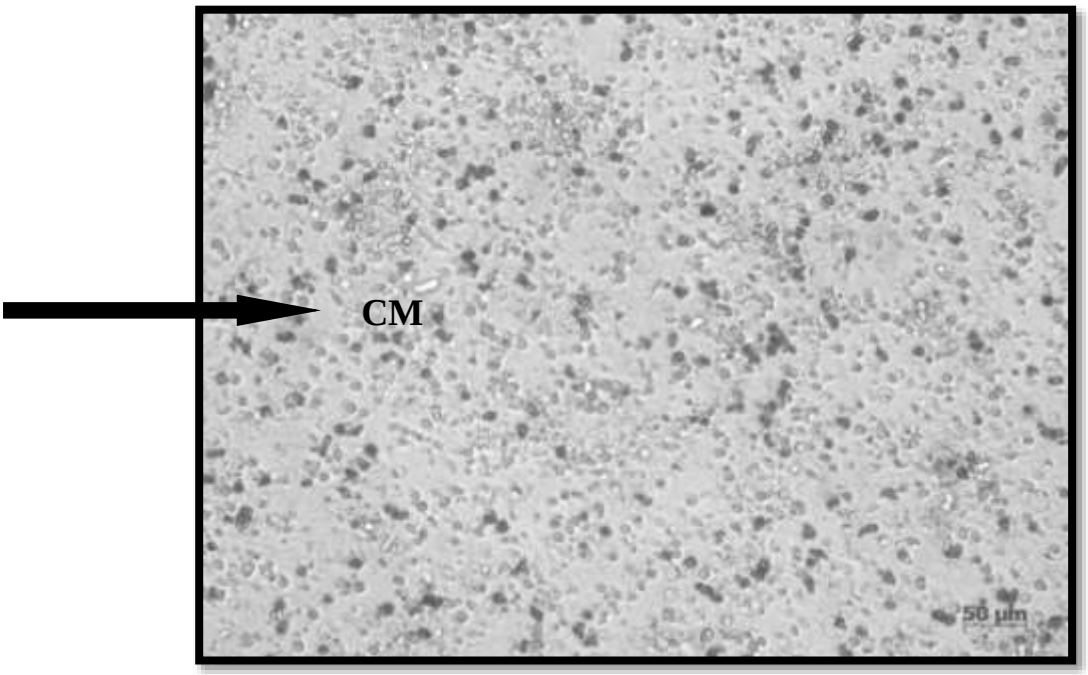


Figure (4-12). Primary cell culture of coelomocytes of sea urchin (*p.lividus*), control group (scale bar=50µm, TEM).CM: Coelomocytes

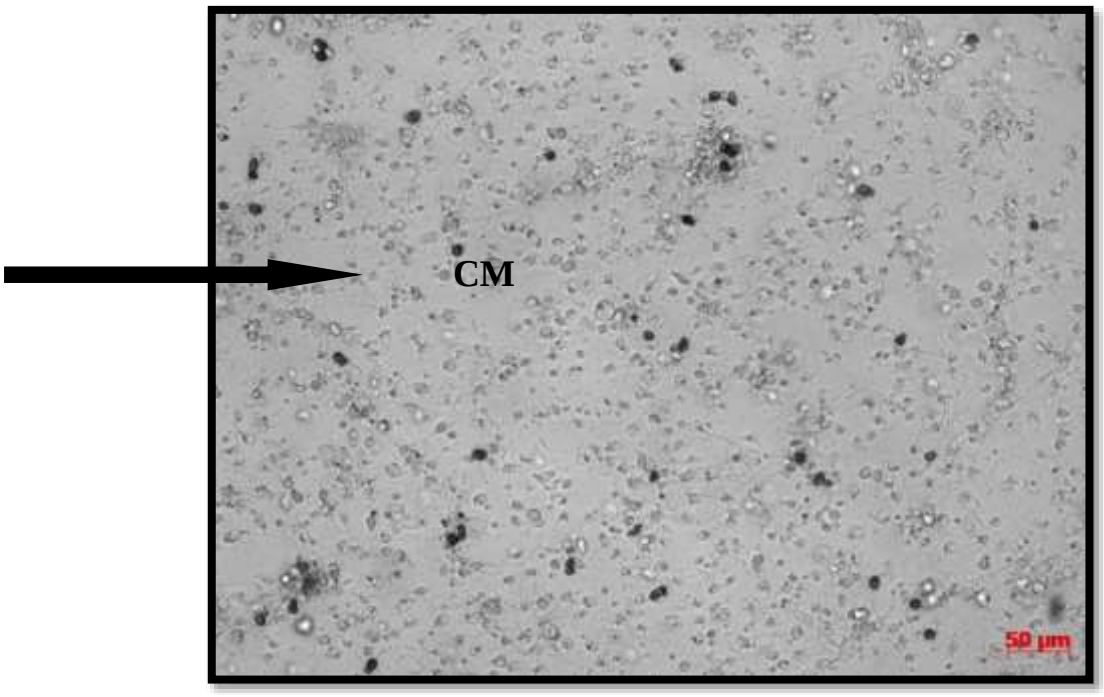


Figure (4-13). Primary coelomocytes cell culture of sea urchin (*p.lividus*) treated with acetone only control group B (scale bar=50µm, TEM) CM: Coelomocytes

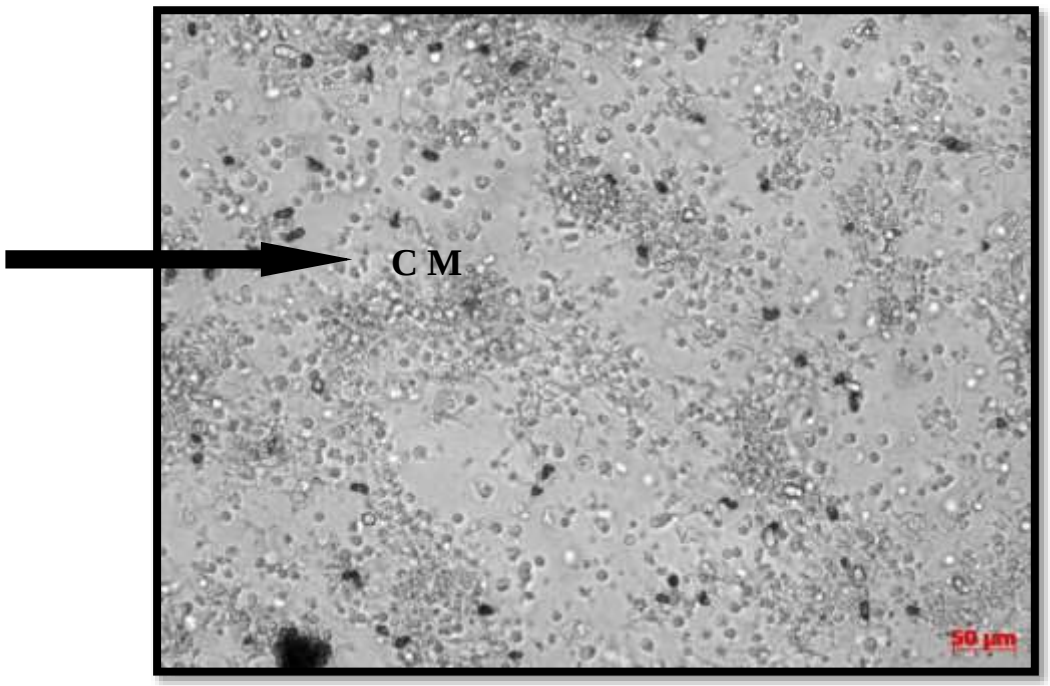


Figure (4-14). Primary cell culture of coelomocytes of sea urchin (*p.lividus*), using L-15 medium combined with 20 pg of E_2, after 6 days of incubation (scale bar=50µm, TEM). CM: Coelomocytes

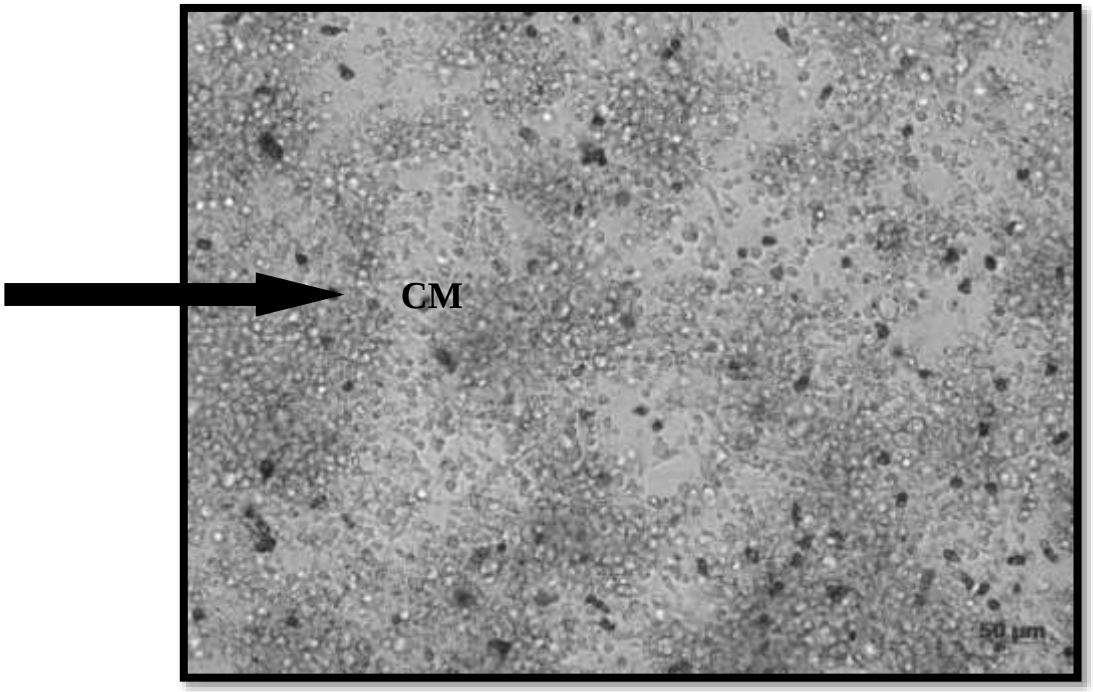


Figure (4- 15): Primary cell culture of coelomocytes for sea urchin (*p.lividus*), using L-15 medium combined with 200 pg of E_2 after 6 days of incubation (scale bar=50µm, TEM). CM: Coelomocytes

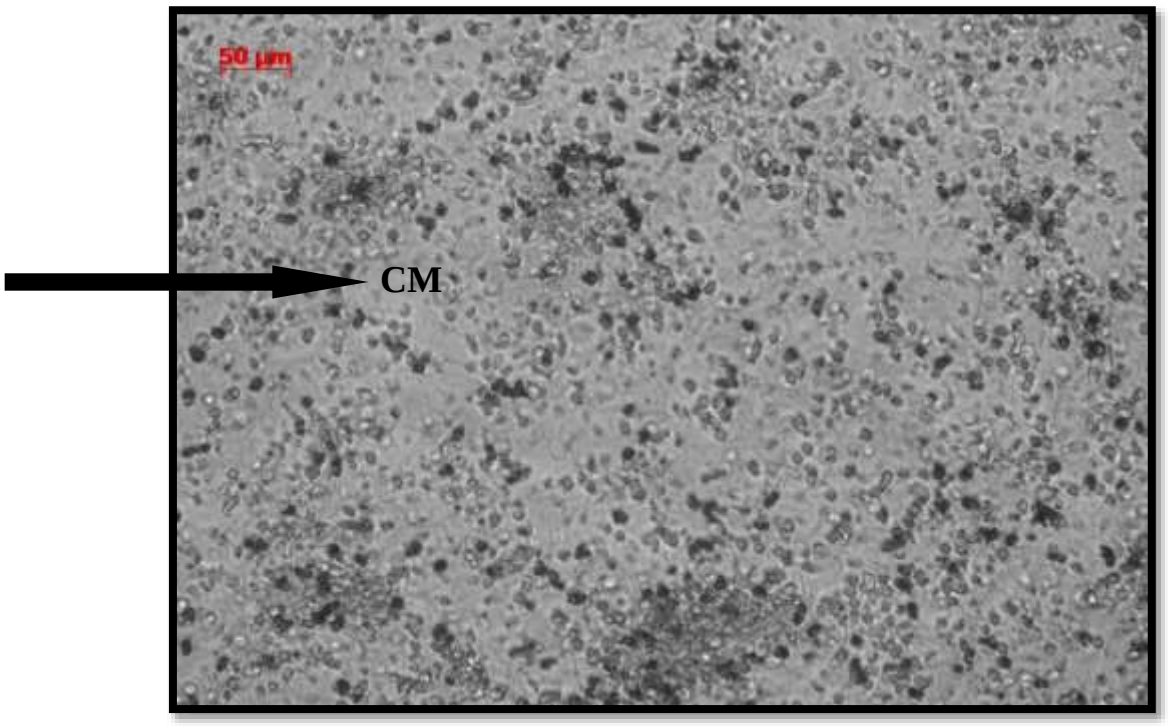


Figure (4-16). Primary cell culture of coelomocytes for sea urchin (*p.lividus*), using L-15 medium treated with 2000 pg of E_2, after 6 days of incubation (scale bar=50µm, TEM). CM: Coelomocytes

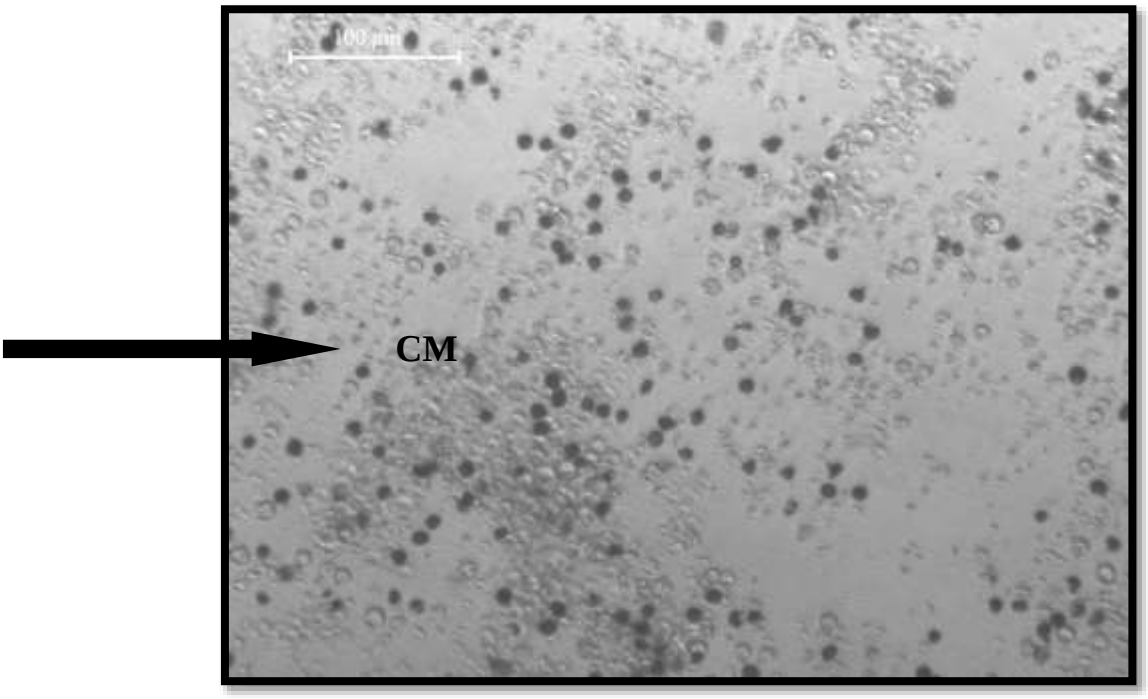


Figure (4-17). Primary cell culture of coelomocytes for sea urchin (*p.lividus*), using MEM medium treated with 2000pg of E_2 after 6 days of incubation (scale bar=100µm, TEM). CM: Coelomocytes

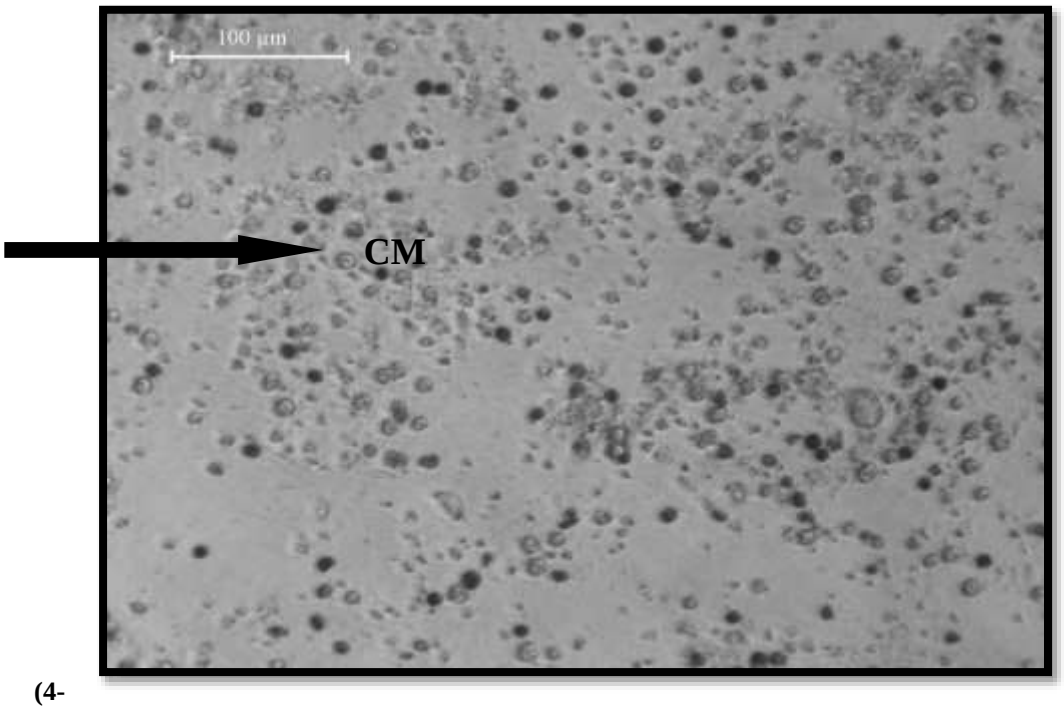


Figure (4-18). Primary cell culture of coelomocytes for sea urchin(*p.lividus*) using M199 medium treated with 2000 pg of E_2 after 6 days of incubation(scale bar=100µm,TEM). CM: Coelomocytes

4-6- The mode of action of Oesradiol (E_2) on the viability of coelomocytes

Steroids are synthesized endogenously and may be involved in the regulation of physiological processes in invertebrate as they are in vertebrates (Dixson, 2001).Previous results supported that sex steroids may have significant physiological roles in the reproduction of invertebrates as they have long been known to have in the invertebrates (Osada *et al*., 1998).

It could be speculated that in the specimens injected with 20 and 200 pg/mL E_2 (i.e. concentrations markedly close from the physiological levels) some metabolic mechanism could have been activated in order to eliminate the E_2 excess and restore the physiological condition, the tested E_2 concentrations (20, 200, 2000 pg/mL) did not cause acute toxicity effects, as further supported by the good health conditions displayed by all the hormonally treated sea urchins (Silva, 2010).

It has also previously been established that oestradiol potentiate 5-HT induced egg release in Japanese scallop and demonstrated that oestradiol has

similar action in the female sea scallop, in the pacific oyster crassosterea *gigas* , it has been shown that oesteradiol increased the concentrations of 5-HT membrane receptors . The increase numbers of membrane receptors may help to potentiate 5-HT induced gamete release. (Dixson, 2001)

Previous work suggested that steroid receptors may be involved in their actions, and the effects of steroids can blocked by analogues known to act as antagonists in vertebrates(Wang,2000).

It has been recognized that sex steroids fundamentally influence the maturation process in different ways: E_2 promoted alteration in the reactivity of the calcium liberation system during cytoplasm oocyte maturation, influencing the typical calcium oscillation during fertilization (Fernandez, 1998).

Sex steroids could bind neuro-transmitter receptors or other membrane components and cause changes in membrane properties (levin, 2001). Other researches demonstrated that lipids and Egg Yolk Extract has a significant positive effect on the viability of *Crassostrea. gigas* heart cell cultures after 2 days, Investigations on the culture of echinoderms cells have proven that invertebrate cells have specific needs for lipids, especially for polyunsaturated fatty acids and sterols (Goodwin, 1991).

As a matter of fact, successful uses of lipid-containing supplements for the culture of marine invertebrate cells have already been reported: whole egg ultra filtrate for *Spisula. solidisim*ma clam heart cells, egg yolk extract for *Mytilus. edulis* mantle cells cholesterol for pearl-producing bivalve mantle cells demonstrated that they might constitute major lipid requirements for these cells and confirm their effects on the viability of *Crassostrea. gigas* heart cell cultures(Cornet, 1992).

Oesteradiol(E_2) involvement in echinoid reproduction has been investigated in a number of studies with an *in vivo* approach. In fact, although the *in vitro* studies allow analyzing the simplest condition, no established cell

culture from marine invertebrates has been developed yet. Nevertheless, good short-term cell cultures can be successfully obtained and employed to explore some processes (Rinkevich, 1999).

On the light of these results we decided to investigate the relationship between the oesteradiol and the viability of coelomocytes appeared to be more influenced by E_2 treatment particularly, we investigate the possible effects of oestradiol on coelomocyte morphology and activities.

In asteroids lipids are mainly accumulated in the pyloric caeca and later on they are transferred to the gonad to be used in gametogenesis while Sea urchins lack pyloric caeca or similar organs and the gonads themselves act as storage organs; particularly most nutrients, including lipids, are accumulated within the gonad somatic cells, the so called nutritive phagocyte (Silvia, 2010)

Previous research suggested that a specific role of E_2 in echinoderm processes could be related to lipid accumulation during the different reproductive stages. For example, in the asteroid *Asterias. rubens* E_2 treatment increased the lipid content in the *pyloric ceaca* and in the sea urchin *L. variegatus* E_2 administration (only in combination with P4) similarly increased lipid percentage in the gonads. Although species-specific differences can be certainly found, these results suggest a hormonal control of lipid incorporation (Fernandez, 1998).

Furthermore, several studies suggested that E_2 could be involved also in protein synthesis: E_2 administration was demonstrated to enhance the rate of protein synthesis in both asteroids and echinoids gonads and, particularly, an E_2 induction of protein synthesis was observed in *S. purpuratus* and *D. excentricus* coelomocytes (Wasson *et al*., 2000a).

The hormone appears to regulate the mobilization of stored yolk precursor and it's translocation into gametes. In both sex in sea urchine, the nutritive phagocytes in the gonads are the main site for storage of nutrients for

gametogenesis and the hormone promoted the accumulation of nutrients by nutritive phagocytes before and during gametogenesis. (Varaksina and Varaksin, 2001).

In echinoid, major yolk glycoprotien in precursor from is synthesized by coelomocytes which are free –wandering cells found ubiquitously in the organs and body cavities, and the precursor is stored in ovarian accessory cells before translocation into the oocytes (Wasson *et al*., 2000b).

Recent investigation show that the E_2 is involved in the regulation of echinoid vitellogenesis, the level of E_2 in the echinoids fluctuate in relation to the reproductive cycle and the treatment of echinoids *in vivo* or ovaries *in vitro* seems that The accumulation of nutrients into the nutritive phagocytes may have been enhanced by steroid through the synthesis of to stimulate vitellogenesis (Lawrence, 2000).

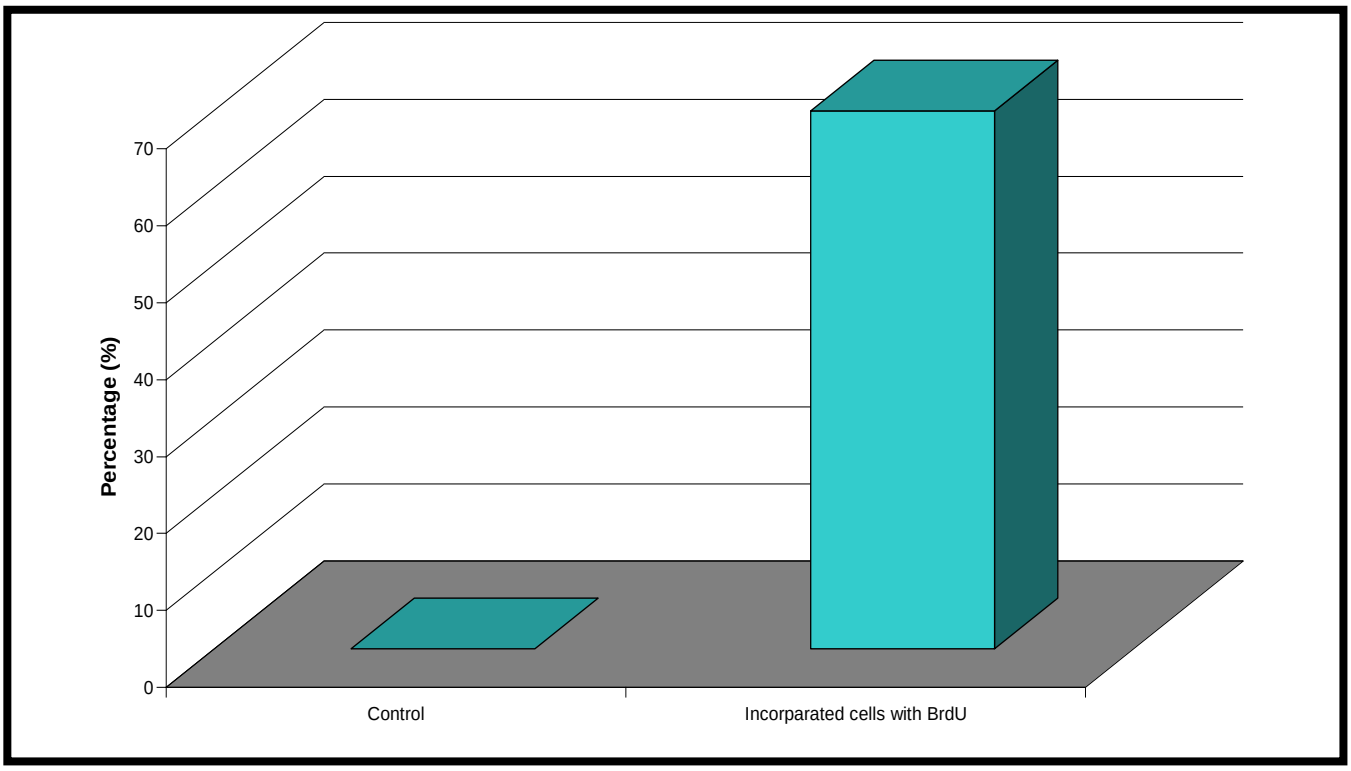
In sea urchin, E_2 may promote biosynthesis of protein and its subsequent mobilization into the gonads, our results confirm that the viability of coelomocytes cultures from *p.lividus* improved when the medium was supplemented with steroids hormone (E_2).

Previous studies on E_2 levels correlated with higher gonad maturative stages in *P. lividus* reported higher level of the hormone in advanced maturative stages (Growing and Premature of *p.lividus*),and oestradiol E_2 involvement in late processes of gametogenesis and suggested that a specific role of E_2 in echinoderm reproduction could be related to the lipid accumulation during the different reproductive stages (Wasson *et al*., 2000b).

4-6: Detection of coelomocytes proliferation.

Cell proliferation seemed to be enhanced in the exposed samples. As indicated by BrdU incorporation studies, extensive cell cycle activity was clearly indicated by BrdU labeling specifically localized at the nuclei of coelomocytes In contrast, in the control group were never

Labelled with Brdu Figure (4-20),(4-21).



Figure(4-19).proliferated coelomocytes of sea urchin(*p.lividus*),the cells incorporated with BrdU compared with control group.

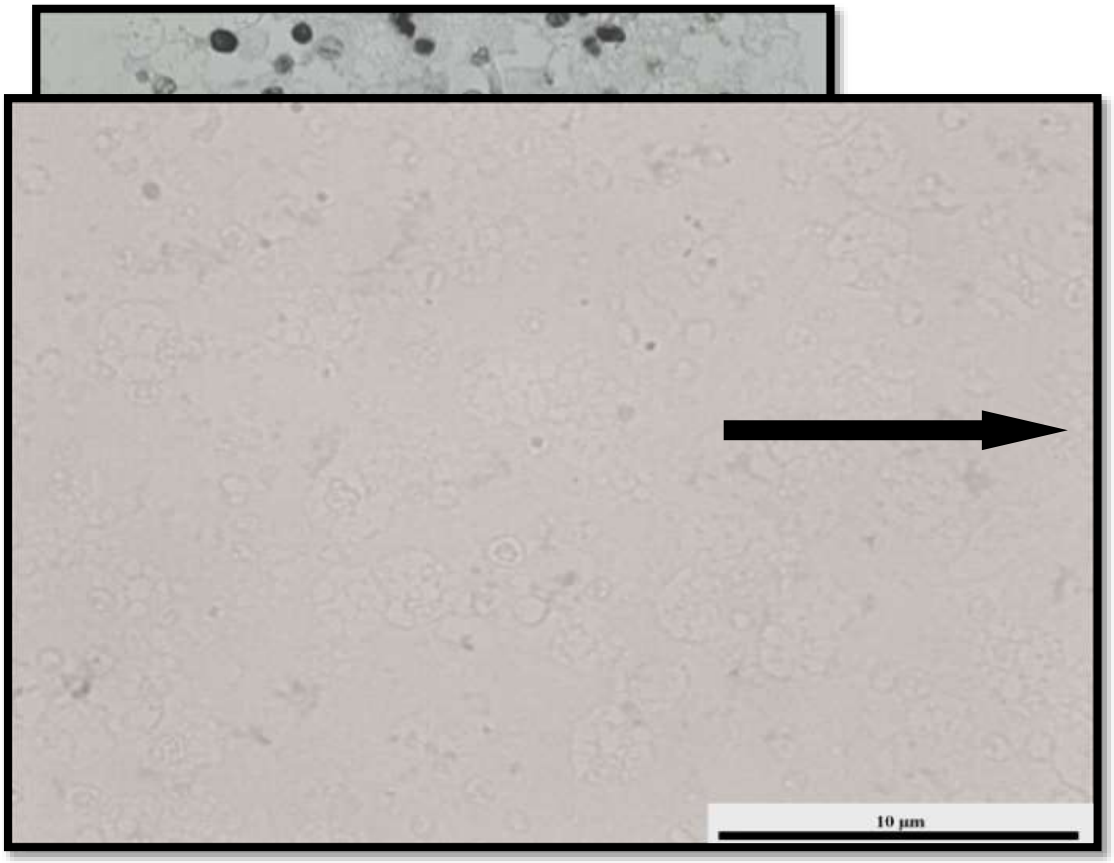


Figure (4-20):BrdU control samples of coelomocytes for sea urchin(*p.lividus*) (without primary antibody) no signal is present(scale bar=100µm,TEM). CM: Coelomocytes

PCM

Figure (4-21) :BrdU treated sample of coelomocytes for sea urchin. The signal is strongly evident (black spots represent nuclei of the proliferating cells)(scale bar=100µm,TEM) .PCM: Proliferated Coelomocytes

4-7 Proliferation of coelomocytes.

The aim of the second part of this study was to employ the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) to label cell cycle activity, Several BrdU labeled nuclei appeared, at this time, no nuclei labeled with BrdU could be detected in control groups.

In view of the intense activity in the epithelium cells and the apparent continued division of labeled cells in tissue left for various periods after the pulse, as shown by the increasing numbers of BrdU labeled nuclei. This would result in the rapid local production of the cells needed for outgrowth of the ablated structures (Chaga *et al*., 1995).

Proliferation phenomena have been described in cultures of marine invertebrates (Fraser and Hall, 1999; Rinkevich 1999, 2004).However, the enhancement of cell proliferation that was observed in the axial organ, coelomic epithelium and Tiedemann body for *A.Rubans* as response to mitotic compounds, suggests that these organs are not only storage sites but also sites of renewal of coelomocytes(Cristiano,2009).

For example, the haematopoietic tissue of crustaceans. It has been described that the distribution of the different stages of haemocyte

development is not homogenous (Johansson e*t al*., 2000).Previous studies have shown that the proliferation rate of cells released from HPT of the Norway lobster, *Nephrops. norvegicus*, was approximately 10% when determined in lobsters not exposed to any pre-challenge (Hernroth *et al*., 2004).

It has been recorded, both by measuring [^{3}H]thymidine uptake and by flow cytometric analysis able to detect the cell cycle phases S, G2 and M, that LPS induces proliferation of circulating haemocytes in the shrimp *Penaeus. japonicus* (Sequeria *et al*., 1996).

In the BrdU method for monitoring cell proliferation in coelomocytes, we applied the same protocol *in vivo*. Different parameters have been modified in the protocols according to the *in vitro* model represented by a pellet of cells (Odintsova, 2005).

To analyze the proliferation cell activity, the method of incorporation (BrdU) was used to determine the level of (BrdU) incorporation in the initial cell suspension agreements with other studies *in vitro* were also performed on representatives of the asteroidean family and were connected the interaction of coelomocytes with pathogen and coelomocytes adhesive abilities (Burker andWalkirs,1999).

In previous study, the primary cell cultures isolated from coelomic fluid of adult *A.rubens* individuals were obtained, after long incubation of *A.rubens* in sea water with high concentration of BrdU (36H, 250um) the proliferative cell, activity increase in the damage zone 7-12 days after one – third of the arm is served the incorporation of BrdU is observed in the

regenerative bud zone at the distal end of each damaged organ (Moss *et al*., 1998).

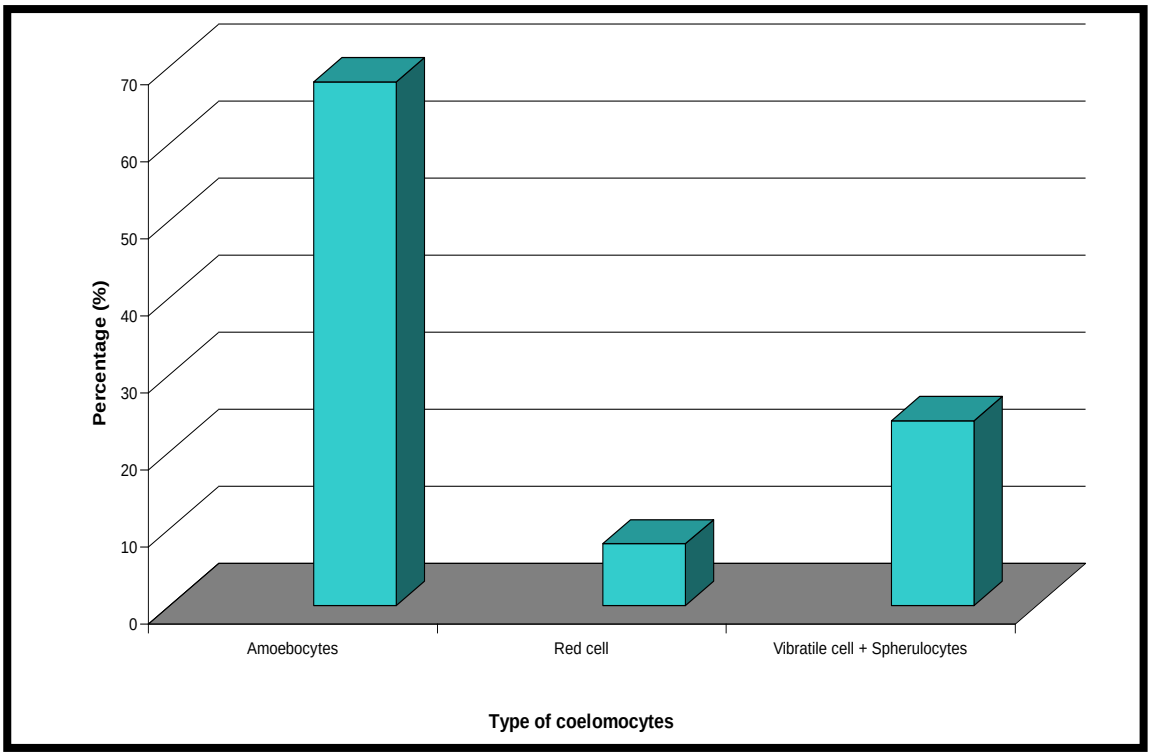
In mammals and fish, there is good evidence that the lymphocytes that proliferate in response to lipopolysaccharide (LPS) are B lymphocytes, and those that respond to the plant lectin concanavalin A (ConA) are T lymphocytes (Sizemore *et al*., 1999).

In invertebrates, which lack the lymphoid cell line, addition of ConA to primary cell cultures of haemocytes has shown effects on cell Attachment and cell proliferation (Lebel *et al*., 1996), and LPS has been used successfully for stimulating proliferation of HPT cells (Van de Braak *et al*., 2002).

4-8-coelomocytes phenotypes and their activities

The density gradient and the positions to which the different types of coelomocytes migrate is shown in (Figure 4-21).The most dense cells, the red spherule cells, migrated to the surface of the 30% iodoxinol layer ,and the vibratile and spherule cells migrated to the 20% iodoxinol layer, while the colorless spherule cells migrated to the 10% iodoxinol layer.

Figure (4-22): coelomocytes subpopulatios using gradient step centrifugation with Iodoxinol.



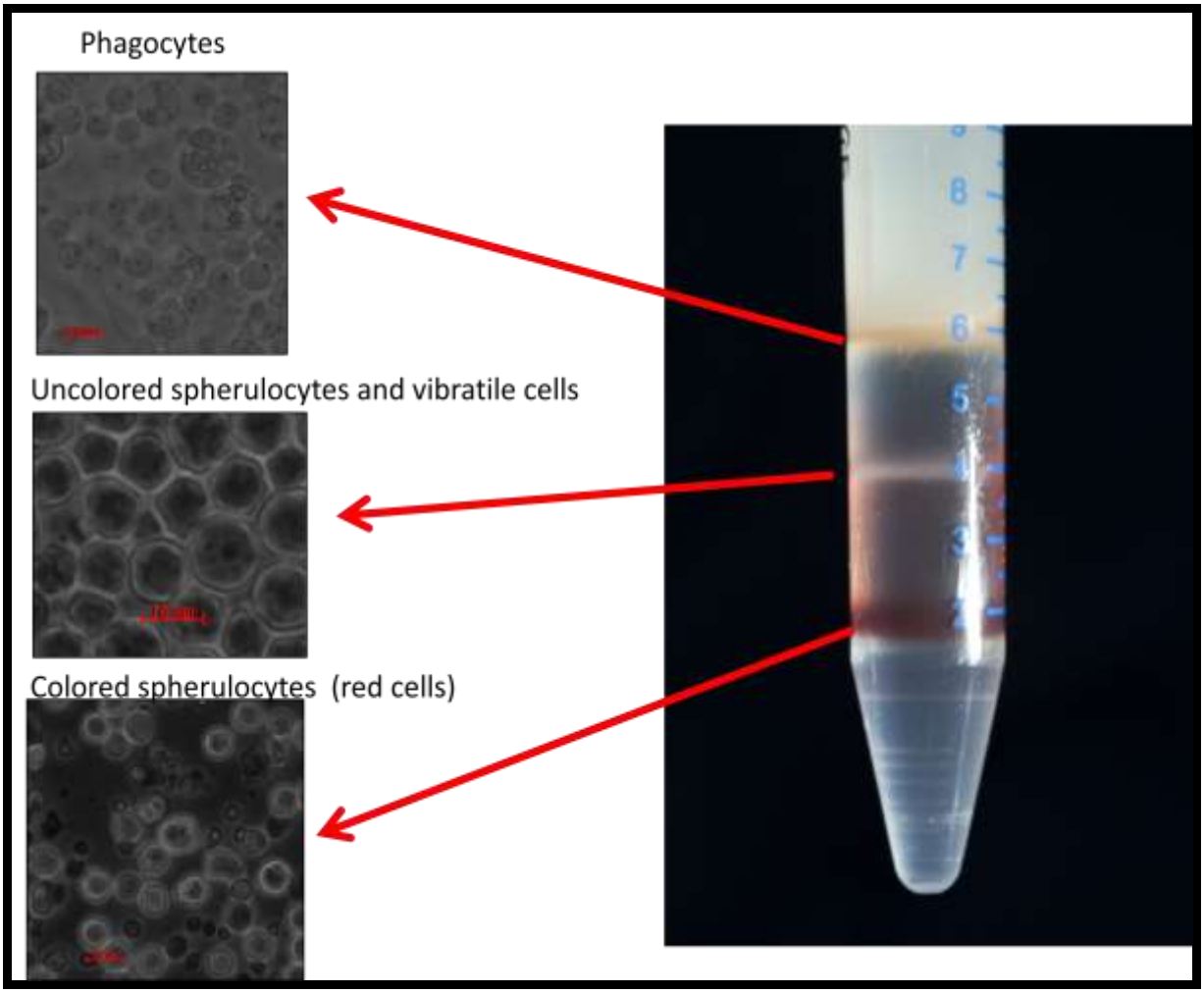


Figure (4-23):gradient centrifugation of coelomocytes appear four different types of coelomcytes; amoebocytes uncolored spherulocytes ,vibratile ,and red cells (scale bar=10µm,TEM).

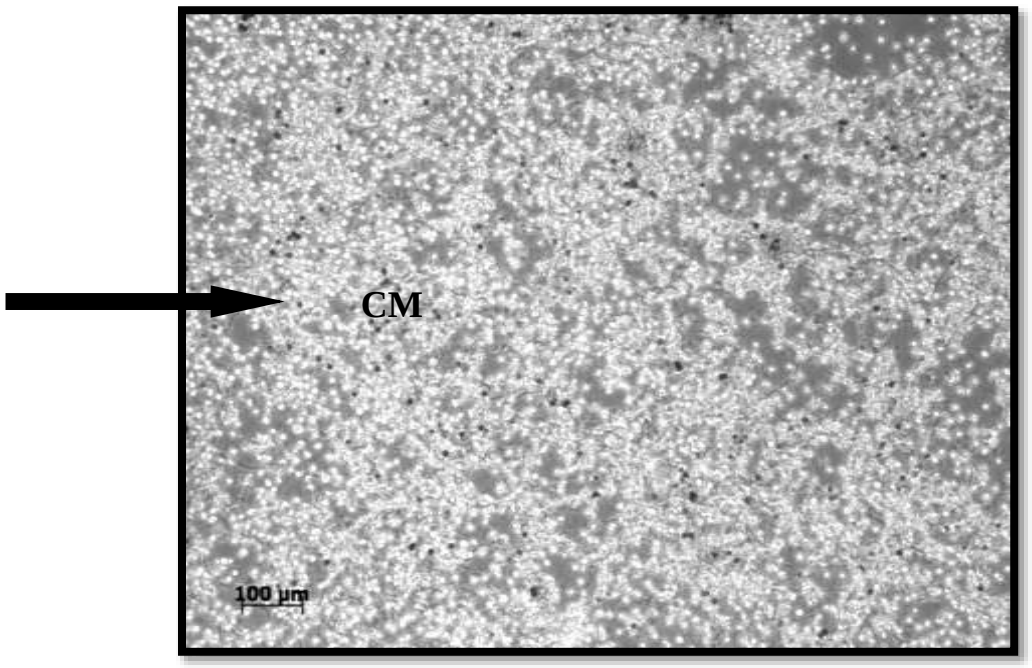


Figure (4-24). Coelomic fluid of sea urchin (p.lividus) contains several million coelomocytes per ml: the bottom of the petri dish is completely filled by coelomocytes (scale bar=100µm, TEM) CM: Coelomocytes

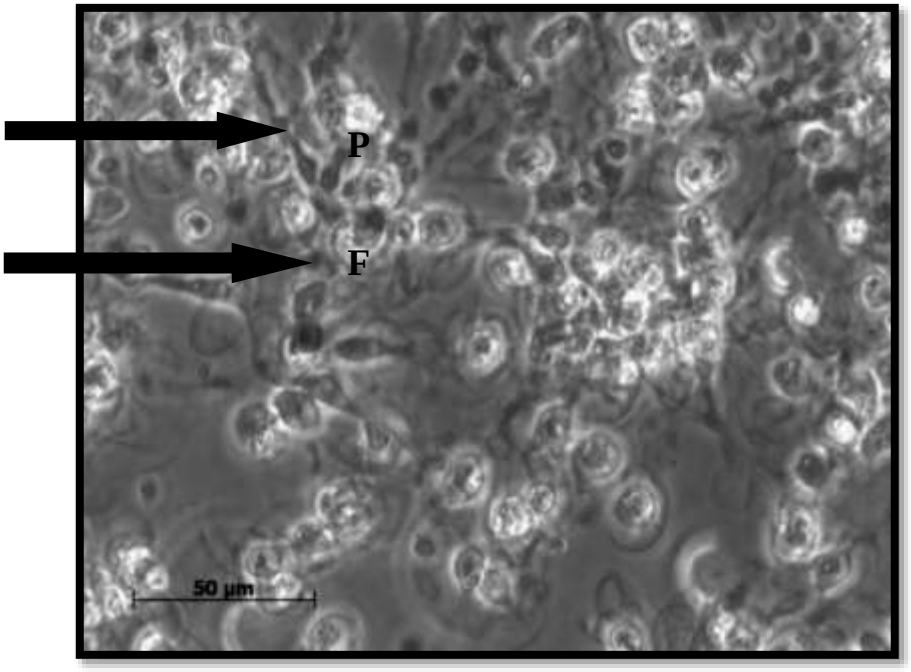


Figure (4-25): Coelomocytes produce lamellipodia/filopodia to adhere the substrate (scale bar=50µm,TEM). P: Petloid, F, Flipodia

Coelomocytes *in vitro* appeared to adhere early to the substrate producing wide lamellipodia with the tipical petaloid form. By employing double staining D/P it was possible to emphasize the wide network produced by coelomocytes connected to each other. (Figure.4-24, 4-25).

4-9- Coelomocytes subpopoulation, morphology and ctivities.

One of the aims of the present research was to characterize the cellular phenotypes of coelomocytes for *p.lividus* that already characterized *in vivo*, by attempting for the first time an *in vitro.* We carried out complementary *in vitro* studies on the echinoid model *p. lividus* which possesses a large amount of coelomic fluid containing several free-wandering phenotypes of coelomocytes (red cells, amoebocytes and vibratle cells) Primary cell cultures from coelomic fluid of *Paracentrotus lividus* have been therefore produced,

In the present study we succeeded to keep viable primary cell cultures of echinoderms (*p.livdus*) at least for two weeks during this period it was possible to provide evidence of different activities and processes related to the cultured cells, In this case, in primary cell cultures, at least four phenotypes are present.

By employing a range of microscopically techniques and protocols In particular, it was relevant to underline that the characterization of phenotypes and the evidence of cellular activities and processes had been obtained by combined employment of different type of analysis, namely light and electron microscope.

L-15 is a complete medium that can stimulate the cells to adhere on the substrate, to observe coelomocytes adhering quickly on the surface and producing large lamellipodia to acquire characteristic “petaloid”

Coelomocytes were quite similar in shape and ultra structure to those described in *A. mediterranea*: on the other hand there probably are substantial

differences in terms of properties because “echinoid coelomocytes” seem to adhere quickly and firmly to substrate and often live in cell suspension (cristiano, 2009).

By DAPI/phalloidin double staining and it was possible to underline that coelomocytes not only firmly adhere on the substrate but take contact to each other forming a real network on the surface. The employment of trypan blue staining method allowed us to establish that coelomocytes can survive in L-15 medium (at least for a tow weeks).

The large number of cells is presents on the substrate surface: interestingly a discrete number of cells appear to have penetrated in the deep extracellular matrix. Cells produce lamellipodia and filopodia , to contact the substrate and each other.

A very preliminary xeno-transplant experiment with cells of expected, crinoids cells adhered weakly if compared to echinoid coelomocytes. These cells can rapidly convert to petaloid and filopodial stages and able to form networks when attached to glass slides (Pinsino *et al*., 2007).

The morphological and biochemical characterization of coelomocytes is, however, still not completely understood, and no reliable, specific cell markers are available. Four sub-populations – phagocytes (previously called amoebocytes), vibratile cells (with flagellum), morula cells (also called haemocytes) and slow-moving cells with an irregular shape (recently called amoebocytes) – have been described (Munoz-Chápuli *et al*., 2005).

High-resolution microscopy was performed to compare the morphology and behavior of circulating coelomocytes and cells released from explants of coelomic epithelium. Evidently, many of the cells migrating from the coelomic epithelium showed morphological similarities to the phagocytes of the coelomic fluid and the released cells were able to form petaloid and

filopodial extending that rapidly could be withdrawn or rearranged. These cells attached to the glass and, when encountering yeast cells, engulfment was observed. At an appropriate density, the cells were also able to form net constructions to trap the yeast, in the same way as observed for the circulating coelomocytes. (Pinsino *et al*., 2007).

Subpopulation of coelomocytes can be purified from total cell suspention, the proportion of each cell type can vary not only among species, but also between individuals of the same species (Gross *et al*., 2000)

According to (Gross *et al* .,199l), the coelomic fluid of echinoids contains four different coelomocyte types including (approximately) 76% amoebocytes, 8% pigmented or red cells, 4% uncolored spherulocytes, and 12% vibratile cells. In the present study we examine similar proportions of P. *lividus* coelomocytes .

The density gradient and the position to which the different types of coelomocytes is migrated to the surface of the iodoxinol, the red cells migrated to the 30% Iodoxional layer the colorless spherule cells and the vibratle cells co- migrated and appeared as mixture on the (20%) Iodoxional layer, the amoebocytes migrated to the 10% Iodoxional layer. Gradient separated coelomocytes into three distinct layers near 10-30% interface, the denser coelomocytes migrated slightly into the 30% iodixinol layer, while the less dense fraction migrated well into 20, 10% iodoxinol layer respectively.

Discontinuous density centrifugation has been used previously to separate morphologically different types of coelomocytes into different fraction employing sucrose gradient, Tow distinct subpopulation of coelomocytes separable by density gradient separation, have been reported previously for *s. purpuratus*, using sucrose gradient (Edd, 1993)

We obtained similar results for p.lividus using iodixinol and microscope examination of the coelomocytes fraction yielded four distinct morphologies. in the coelomic fluid of the sea urchin four distinct cell types

have been identified ;amoeboid phagocytes ,red spherule cells ,colorless spherule cells and vibratle cells.

Conclusions:

From the results obtained in this study, it can be concluded that:

- Among the basal media, L-15the antibioticsand is a suitable medium for *in vitro* culture of coelomocytes and gonad cells from sea urchin *p. lividus.*
- The addition of oestradiol hormone (2000pg /ml) has a positive effect on cells viability and growth improvement.
- L-15 medium can promoted better coelomocytes adherence and forming of lamellipodia/filopodia
- the BrdU induced Proliferation coelomocytes shown by the increasing numbers of BrdU labelled nuclei.
- characterizing coelomocytes phenotypes *in vitro* .

RECOMONDATIONS

This work represents a good starting point for further studies on primary cell cultures in echinoderms. The next important steps in this promising branch of research appear to be related to:

- improve the primary cell culture setting up, with particular reference to increasing the cell amount *in vitro*.
- obtain different cell cultures of selected cell lines, in order to study separately individual phenotypes.
- Analyzed in detail migration and dedifferentiation processes, with particular reference to coelomocytes.
- develop and establish appropriately the original method utilizing to achieve really established and long-term cell cultures for *in vitro* systems of marine invertebrates.

- it is apparent that there is need for further work to develop ideal medium for gonad specific tissue in the culturing of *P. lividus* cells *in vitro*, in

order to enhance the growth of both germinal and somatic cells. Certainly, this study has demonstrated the viability of primary cell cultures of *P. lividus*, which should have value particularly in eco-toxicology investigations and in studies concerning echinoderm reproductive biology.

- The presence of proliferation processes in coelomocyte cell cultures open a wide range of perspectives among which the possibility to obtain a secondary cell culture could be the most intriguing issue.

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