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Melanization stimulating factors in the integument of the *Mugil cephalus* and *Dicertranchus labrax*

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Summary. The pigment pattern expression resides in the chromatoblasts of the embryonic skin. The differentiation of these chromatoblasts is influenced by specific local factors such a melanization inhibiting factor (MIF) and a melanization-stimulating factor (MSF). We reveal the presence of these factors by means of a series of experiments on the skin of the marine species of fish *Dicertranchus labrax* and *Mugil cephalus*, each with different pigment pattern, the former having a light skin and the latter a darker one. Media conditioned by exposure to dorsal and/or ventral skin, stimulates the melanization of *Xenopus laevis* neural crest cells throughout a 3 day assay period. Similarly conditioned culture media tested on B16-F10 murine malignant melanocytes, revealed a considerable influence in enzymatic activities: dopachrome tautomerase (DCT), tyrosine hydroxylase and dopa oxidase. The use of media in a dose response basis suggests that the conditioned media may contain both melanophore stimulating and inhibiting factors. The results obtained may actually reflect the resultant activity of the two factors present.

Key words: Melanogenesis, Stimulating-factors, Enzyme-activity, Teleosts

Introduction

The striking and varied pigmentation patterns of poikilotherms have long attracted interest and an understanding of their biological and physical bases has been sought after for many years. Despite these efforts, substantial definitive knowledge about the development and maintenance of pigmentation pattern is lacking, although it is obvious that the ultimate expresssion of specific patterns depends upon the distribution of specific pigment cells (Bagnara, 1998).

All pigment cell types in vertebrates originate from the neural crest and are determined (in the embryonic sense) either in situ or by extrinsic factors encountered during migration or after their final destination is reached (Derby, 1982; Bagnara, 1987; Perris et al., 1988; Campbell, 1989; Hou, 1999). The matter of pattern formation was thought to be influenced by the tissue environments in which chromatoblasts differentiate. It is likely that the dorsal skin plays a critical role in inducing the differentiation of neural crest cells towards pigment cells (Derby, 1982; Perris and Lofberg, 1986; Perris et al., 1988; Johnson et al., 1992; Mangano et al., 1992).

The effect of extracellular matrices and growth factors on potential pigment cells has been reviewed (Erickson, 1993; Frost-Mason et al., 1994). The cellular basis for pigment pattern formation must be considered in terms of the effects of these determinants on the migration proliferation and differentiation of chromatoblasts (Fukuzawa and Obika, 1995). It has long been proposed that the integument contains factors which regulate pigment pattern expression. The experimental data has supported the alternative view that the pigment pattern expression resides in the embryonic skin and that chromatoblasts are influenced by specific local factors that influence the differentiation of these chromatoblasts (Bagnara et al., 1979). These factors may include elements of the extracellular matrix and local factors present in the integument such a melanization-inhibiting factor (MIF) and a melanization-stimulating factor (MSF). MIF and MSF may be major elements in controlling the expression of pigmentation patterns. MIF was described in amphibia by Fukuzawa and Ide (1988), and MSF was described in frog skin (Mangano et al., 1992). This line of investigation was extended to fishes, and revealed the presence of MSF in the skin of the freshwater *Ictalurus punctatus* (Johnson et al., 1992; Zuasti et al., 1992), and in the marine species *Sparus auratus* (Zuasti et al., 1993).

This work is part of a series of investigations that are designed to utilize a relatively new approach to studying the mechanism of pigment pattern formation. Questions arise about the presence of MSF activity in other marine species of fish such as *M. cephalus* and *D. labrax* because of their highly distinct pigmentary pattern *M. cephalus* is darker than *D. labrax*. The results of a series of experiments using crude extracts of dorsal or ventral

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skin of these marine species were analyzed with the *Xenopus* neural tube explants and with B16-F10 malignant melanocytes measuring all melanin enzyme activities: tyrosine hydroxylase, dopa oxidase, dopachrome tautomerase and melanin content, to detect the possible presence of MIF or MSF activity.

Materials and methods

Materials

The chemicals used, obtained from either Sigma (St. Louis, MO), Merck (Darmstadt, Germany) or Panreac (Barcelona, Spain), were of the highest commercially available purity, and were used without further purification.

Culture media, fetal calf serum, Hanks' isotonic salt solution, and other reagents for malignant melanocyte culture were purchased from Flow Lab (Irvine, Scotland). L-(3,5-^{3}H) -tyroxine (specific activity 58.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA). All solutions were prepared with bidistilled, deionized water, with a resistivity higher than 10 M cm.

For the experiment ten healthy adult specimens of each species were used: *M. cephalus* (Mugilidae. Mugiliformes) and *D. labrax* (Serranidae, Perciformes). They were obtained through the generosity of the Oceanographic Institute (Murcia, Spain).

Preparation of conditioned media

Ventral and dorsal skin of the *M. cephalus* and *D. labrax* were chopped into small pieces and placed in the growth media appropriate for either the neural tube explant of *X. laevis* or for the culture of B16-F10 murine melanoma cells, at a concentration of 0.07g of skin per ml of medium, and shaken for 1hr at 25 °C. The conditioned media were sterilized by filtration through a millipore membrane (0.45 μm). The initial concentration of conditioned medium was taken as 100%.

Neural tube explant system

Neural tubes of stage 22/23 *Xenopus* embryos (Nieuwkoop and Faber, 1956) were explanted and used as the source of neural crest cells for the in vitro experiments as described by Fukuzawa and Ide (1988). Essentially, the epidermis, somites, and notochord were removed from *Xenopus* embryos that had been treated with 0.1% collagenase for 30 min. Individual neural tubes were then cut with tungsten needles at the level of the seventh somite and the caudal half of each neural tube was cultured at 25 °C in a sitting drop of about 100 μl of either control or conditioned Steinberg's balanced salt solution (BSS, pH 7.4) (Jones and Elsdale, 1963) on a tissue culture dish (Falcon, No. 3002; Oxnard, CA). The BSS used for culture contained 0.02 mM tyrosine, penicillin (Sigma) at a concentration of 100 IU/ml, and streptomycin (Gibco, Grand Island, NY) at 100 μg/ml. The cultures were observed daily and counts were made of the total number of cells emigrating from the neural tube explants and the number of melanized cells. Counts were discontinued after 3 days, because complete detachment occurs after 4 days in culture. Newly differentiated melanophores were easily identified with bright and phase-contrast optics by the presence of dark melanosomes, which was earlier confirmed by electron microscopy (Fukuzawa and Ide, 1988). In order to ascertain the presence of factors in *M. cephalus* and *D. labrax* skin that might affect pigment cell expression, the neural tubes were cultured in BSS conditioned by exposure to dorsal or ventral skin. Conditioned BSS was diluted with control BSS to obtain the desired concentrations to be used in the cultures.

Cell culture

B16-F10 melanocytes were maintained in minimum essential medium (MEM, Eagle modified with Earle's salts) supplemented with 2mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. Cells were ground in 75 cm^2 flasks (Nunc, Roskilde, Denmark), at 37 °C, in a water saturated with 5% CO_2 atmosphere. The cells underwent routine passages every 4 days, with media changes every 2 days. They were harvested by incubation in a solution of 0.1 mg/ml trypsin with 0.2 mg/ml EDTA in pH 7.2 Hanks' without Ca (II) and Mg (II) for 5 min, with gentle shaking of the flasks. Cells were pelleted by centrifugation at 1,000 rpm for 10 min at 22 °C in a Heraeus Minifuge T. The pellet was resuspended in pH 7.2 Hanks' solution and cells were counted using the trypan blue exclusion test and a haemocytometer. A duplicate set of flasks was always used for every experiment. The experiments were carried out at least three times with similar results.

Enzyme assays

Prior to assaying enzymatic activities, cells were disrupted by resuspending them in a hypotonic medium (10 mM sodium phosphate buffer, pH 6.8 containing 1% Brij-35 and 0.1 mM of phenyl metylsulfonyl-fluoride) followed by sonication in an ice-cold water bath for 5 min. After centrifugation at 13,000g for 10 min in an Eppendorf centrifuge, the supernatant was used to measure enzymatic activities and protein content, whereas melanin was quantified in the nonsoluble pellet.

Tyrosine hydroxylase assays were carried out according to Jara et al. (1988) using L-(3-5-^{3}H)-tyrosine (0.05 mM containing 0.5 μCi per assay) as substrate. One unit of tyrosine hydroxylase activity was defined as the amount of enzyme that catalyzes the hydroxylation of 1 pmol of L-tyrosine/min at 37 °C.

Dopa oxidase activity was measured spectrophotometrically according to Winder and Harris (1991), by using MBTH, yielding a dopaquinone-MBTH pink

adduct followed at 500 nm. One unit of this activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of L-dopa/min at 37 °C.

Dopachrome tautomerase (DTC) activity was measured by following DHICA formation from L-dopachrome by HPLC, according to Palumbo et al. (1987); L-dopachrome was prepared by the stoichiometric oxidation of L-dopa by sodium periodate (Aroca et al., 1990). One unit of DCT was defined as the amount of enzyme that catalyzes the transformation of 1μmmol of L-dopachrome/min at 37 °C.

Melanin and protein determination

The melanin content of the pellet was quantified according to the method of Lotan and Lotan (1980), by measuring the absorbance of 400 nm of melanin digested with hot 0.85 M potassium hydroxide at 90 ºC overnight, using synthetic melanin as the standard. Protein estimation was performed by the method of the bicinchoninic acid (Smith et al., 1985), using a kit from Pierce (the Netherlands).

Results

BSS media, conditioned with either dorsal (DCM) or ventral (VCM) skin of *M. cephalus* at concentrations of 10%, 25% and 50%, had a stimulatory effect on the melanization of neural crest cells of *X. laevis* embryos after three day treatment. Such stimulated cells doubled the number of dendrites per cell. In fact, in some cases 90% melanization was observed within the first 24 hours. That was significantly higher than that of the BSS control. The highest stimulation was at 25% concentration with both DCM and VCM conditioned skin. In all concentrations of both dorsal and ventral media, the unmelanized cells were bipolar (Fig. 1a), whereas the melanized cells were more dendritic (Fig. 1b). In the BSS control, the melanized cells possessed an average of only 2.5 dendrites, but melanized cells in conditioned media, with either dorsal or ventral skin, had 3 to 3.5 dendrites per cell.

Regarding *D. labrax*, the effects showed more of a concentration dependence for ventral skin, a behavior very similar to that reported for *M. cephalus*. VCM was stimulatory at 10% and 25% concentrations. It also appears that 10% VCM was sufficient to achieve maximum stimulation, which is taken to be about 95% since the activity of 25% VCM was not greater than 10%.

Incubation of B16-F10 murine melanoma cells with MEM conditioned by *M. cephalus* dorsal skin (DMc) manifested an increased presence of melanin granules in the cytoplasm of these melanoma cells (Fig. 2). This incubation resulted in an increase in dopachrome tautomerase (DTC) (Fig. 3), tyrosine hydroxylase (Fig. 4) and dopa oxidase (Fig. 5) activities after 24 h incubation, the dorsal culture showing a large increase after 24 h which fell by 48 h. However, the melanin content decreased at 24 h and increased after 48 h incubation (Fig. 6). With ventral skin (VMc), there was a decrease in all enzymatic parameters at short exposure, but a significant increase in tyrosine hydroxilase and dopa oxidase over a longer time.

Concerning the effects on the melanogenic parameters in B16-F10 mouse melanoma cells after 24h incubation with MEM conditioned with *D. labrax* dorsal skin (DDl), there was a significant slight fall higher than that of *M. cephalus* results (Figs. 3-5). The earliest observations (24 h) showed a slight increase in melanogenic activities for the dorsal cultures and a decrease in activities for the ventral ones. The melanin content dropped gradually in the dorsal cultures, while in the ventral ones (VDl) we saw a sharp fall followed by an increase in melanin content after 48 h (Fig. 6).

In all cases, cell viability was not modified after 24 h or 48 h treatment (Fig.7).

Discussion

The present study is one of several that we have undertaken using a novel direct approach of looking for intrinsic pigment cell stimulating or inhibiting factors in dark or light skin, respectively.

With the neural tube explant system, it was observed that basic salt solution (BSS), conditioned with either dorsal or ventral *M. cephalus* and *D. labrax* skin, induced increases in neural crest migration and melanization. The melanization-stimulating activity of the conditioned media was shown to act in a dose-response manner. Althought both were active, dorsal skin seemed to be more active than ventral skin. The use of both media, on a dose response basis suggests that there are both melanophore stimulating and inhibiting factors present.

Since MSF activity has also been found in both the dorsal and ventral skin of fishes (Johnson et al., 1992; Zuasti et al., 1992, 1993), it is attractive to consider that an interplay between MIF and MSF in specific areas of the skin play a major role in the determination of the specific pigmentation pattern expressed.

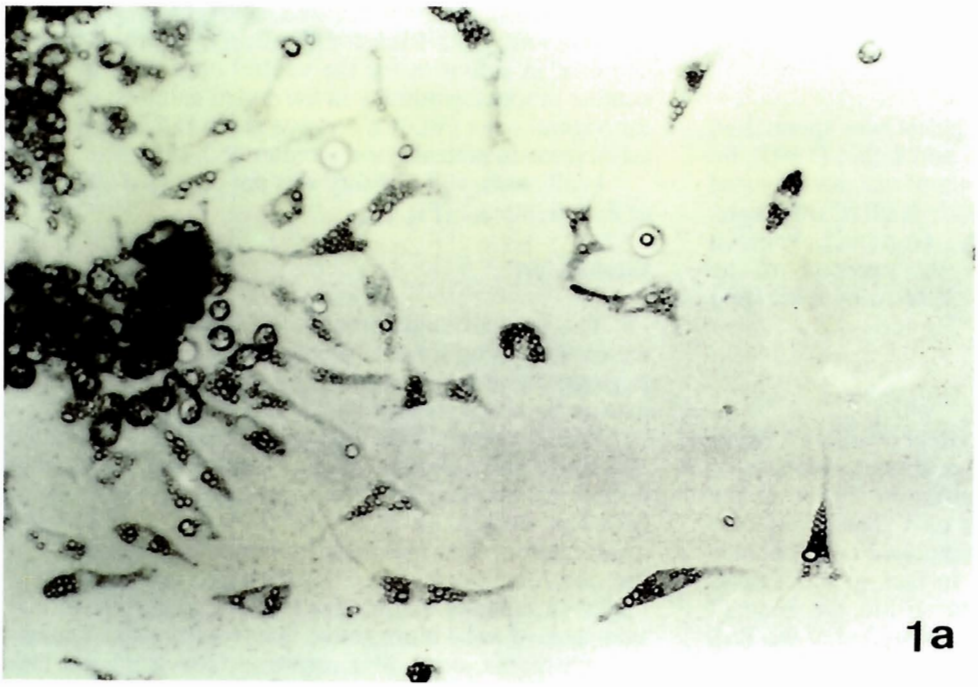
The putative MIF and MSF molecules are presumably produced by cellular elements of the environment in which the pigment cells pass through or are found. In contrast, it is possible that patterns of migration and expression are influenced by molecules produced by the pigment cells themselves (Bagnara, 1998), or by the influence of factors specifically provided by tissues (Hou, 1999).

The fact that the skin of both regions possesses significant MSF activity is consistent with the fact that there are numerous melanophores in both areas. Presumably, a given level of intrinsic MSF would be necessary to support melanophore differentiation and continued expression (Mangano et al., 1992). The more intense level of melanization in the dorsal skin could be explained by a higher local MSF content.

The differences between the MSF activity in VCM

and DCM is much less marked than is the actual pigmentation of this species, wherein the dorsum is almost black and the ventrum is much lighter. At present, this apparent paradox can not be explained, but the fact remains that potent MSF activity is present in both regions of the skin when assayed in the *Xenopus* neural tube explant system. The conditioned media may contain both MSF and MIF molecules and the results obtained may actually reflect the resultant activity of the two factors present.

The conditioned media stimulated an increase in dendrite number. It seems that this increase in dendricity over the typical bipolar condition of neural crest cells in BSS represents a manifestation of well being and that these dendritic cells are perhaps in better physical condition than are the bipolar cells. In this regard, it is interesting that there is a pronounced correlation between melanization and an increase in dendrite



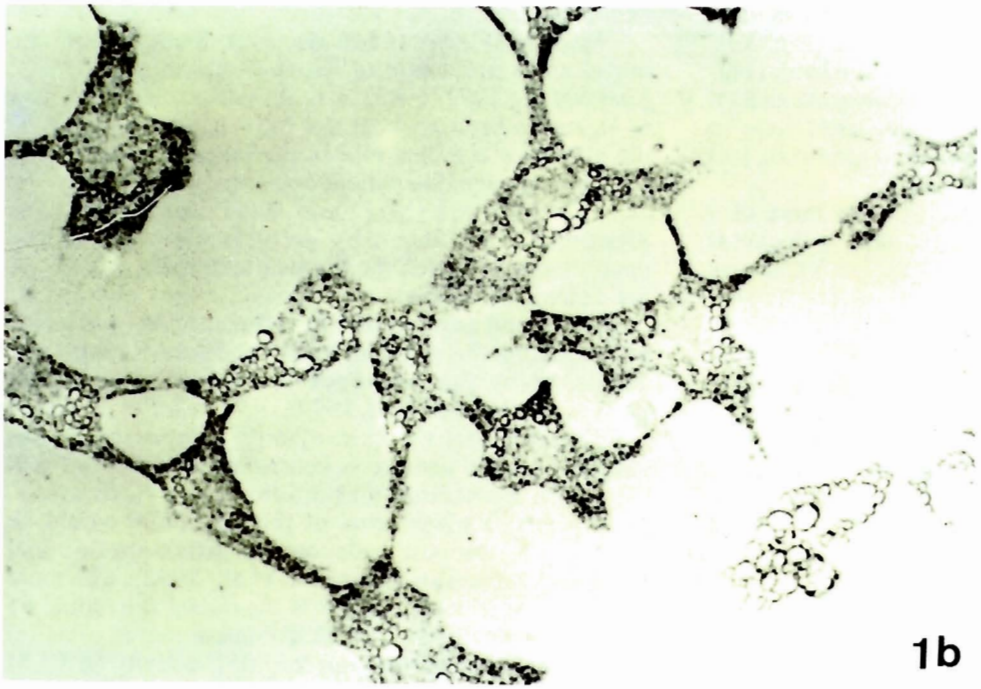


Fig. 1. Neural crest cells emigrating from a *Xenopus* neural tube 2 days after explantation in *M. cephalus* 25% DCM. **a.** In control BSS, neural crest cells are predominantly bipolar, either melanized or not x 400. **b.** In 25% DCM the emigrating cells are fare more dendritic and are melanized. x 1,000

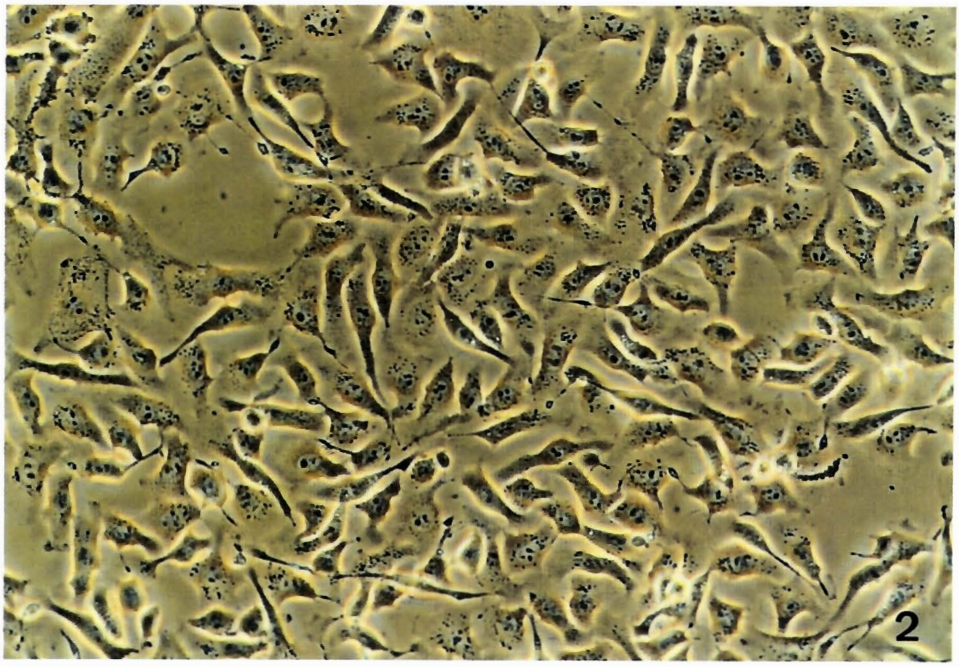


Fig. 2. Phase contrast illumination showing B16-F10 murine melanoma cells after 24h in culture. Cells exposed to *M. cephalus* 50% DCM showing numerous dark granules of melanin. x 400

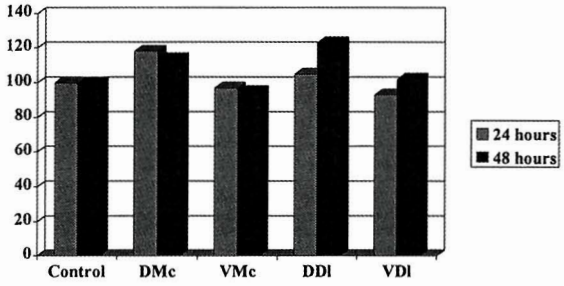


Fig. 3. Dopachrome tautomerase activity (%)

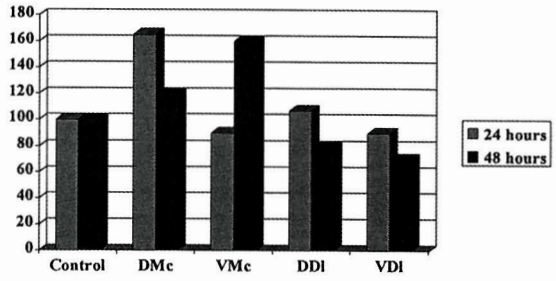


Fig. 4. Tyrosine hydroxylase activity (%).

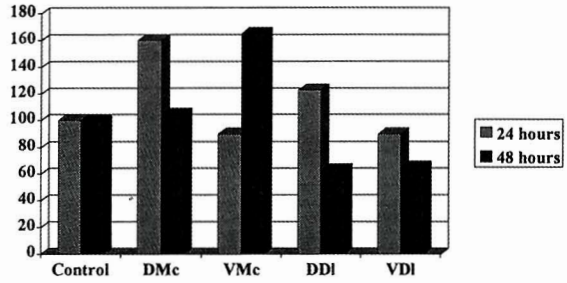


Fig. 5. DOPA oxidase activity (%).

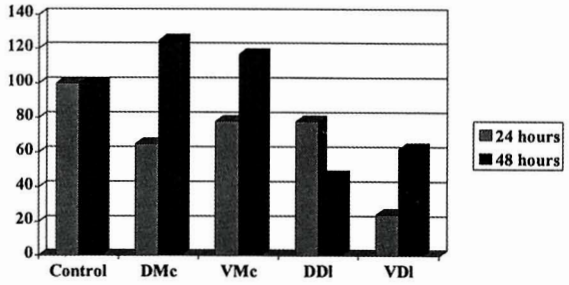


Fig. 6. Melanin content (%).

number (Zuasti et al., 1992, 1993). Because the conditioned media from *D. labrax* and *M. cephalus* also evoke an increase in dendrite number, it is probable that the active factor of both species are similar. Since MSF activity has also beeen reported for the skin of other vertebrates, notably the frog (Mangano et al., 1992) and the Long/Evan rat (Johnson, 1993), it seems likely that MSF molecules are widely distributed in the skin of all vertebrates and may play an important role in the expression of normal and abnormal pigmentation in all vertebrate species from fishes to human (Bagnara, 1998). A limitation of the neural tube assay is that the parameters of melanization that it measures is only a morphological one and thus we do not know from this assay just which events in melanogenesis were affected by the conditioned media. Fortunately, the enzymatic

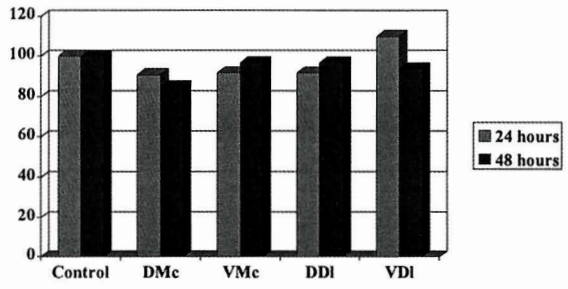


Fig. 7. Cell viability (%).

determination on the murine melanoma cells provided a resolution to this problem. The three important sequential enzymatic steps in melanogenesis are all stimulated by the conditioned media in a dose dependent manner and these activities lead to a final increase in the amount of melanin present in the melanoma cells.

The results of the present investigation provide strong evidence that there are intrinsic pigment cell stimulating factors in the integument of *D. labrax* and *M. cephalus* and that they strongly infuence or even determine, the pigment pattern of these species.

In conclusion, it has been demonstrated in this study that in the skin of *D. labrax* and *M. cephalus*, there are factors that influence the differentiation of the neural crest cells into melanophores. There have been demonstrations too, that these factors influence the enzymatic activity of B16-F10 melanoma cells. This suggests that the determination of pigment pattern may be a result of interactions between MSF and MIF.

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