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Analysis of Fibroblast Growth Factor Influence on Growth and Developmental Potential of Rat Foetuses in the *In Vitro* Culture Model

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ABSTRACT

The fibroblast growth factor's (FGF) influence on the growth and differentiation of 8- and 9- day-old rat foetus has been studied, whereas foetuses were grown in an in vitro culture model. Proliferation was analysed by the expression of proliferating cell nuclear antigen (PCNA). It was established that the usage of FGF in the first period of the culture lowers the growth no matter the foetus age at the moment of culturing and no matter whether it is a medium with or without a serum. If FGF is applied in the second culture period, it also lowers the growth, however younger foetuses in the in vitro culture model are more sensible to FGF negative influence.When FGF was applied in a lower concentration the growth of whole foetuses was improved in the in vitro culture model, which shows that the FGF influence on growth depends on the concentration. Stereological analyses have been done and showed that, in the in vitro culture model, FGF has no influence on proliferating cartilage tissue, but it stimulates the survival of nervel tissue cells. It has been shown that the quantitative research of growth processes in cultivated foetuses can precisely be done by combining classic methods of measuring whole foetus diameters and analysing the expression of proliferating antigen.

Key words: *FGF, embryo, rat, development*

Introduction

Fibroblast growth factor family (FGF) is a family of polypepthide growth factors, which induce various biological processes in cells of both mesodermal and neuroectodermal background[1]. Their tissue distribution as well as their preservation during the evolution point to the importance of their physiological function[2]. Biological activitiy of this family has also been established in the foetus growth as well as in the grown organisms. Family members of FGF signal molecule during embryonic development take part in the process developmental regulation such as proliferation[3], migration[4], differentiation[5] and they are important factor in the pathology of different human illness[6,7]. In a grown organism they regulate different physiological processes such as tissue rebuilding[8], apopthosis[9] and angiogenesis[10]. This research includes the study of rat foetus growth cultivated in an *in vitro* culture model in different chemically defined media, and the FGF influence on their growth and differentiation of various tissues. To estimate the proliferation morfologically a proliferating cell nuclear antigen (PCNA) was used. Detecting the PCNA protein by immunohistochemical methods proved appropriate for studyng the proliferation in both human and animal tissues[11–13].

Material and Methods

In vitro culture

Experiments were performed on pregnant Fischer female rats on different days of gestation. Adult (three--month-old) females were mated with males of the same age overnight. Vaginal plug designated day 0 of pregnancy. Female rats were killed after 9, or 10 days of pregnancy. Reichert's membrane were removed, extraembryonic parts were cut off at the level of the amnion and discarded. The embryos were placed individually on a lens

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paper supported by a stainless steel grid in a tissue culture dish (Falcon No. 3037, Becton Dickinson, Oxford, UK). Medium was added to the dish to wet the lens paper, and embryos were grown at the gas-liquid interface. Eagle's Minimal Essential Medium (MEM) with Hank's balanced salt solution wad used as the baasl medium. Rat serum in MEM (50%) was used for serum-supplemented control cultures. Serum was obtained from the blood drawn from aortas of adult male Fischer rats, which was immediately centrifuged. Serum was inactivated at 56°C for 30 mn and sterilized through a 0.22 μm filter. Fibroblast growth factor from the bovine pineal gland (Sigma F-1881) was added to MEM in concentration of either 12,5 ng/ml or 100 ng/ml. Human apotransferrin (Sigma T-4515) was used in in concentraton of 50μg/ml. The culture medium was changed every other day during the culture period. To monitor growth, the major and minor diameters of the explants were measured on various days of culture by using an eyepiece micrometer.

Histology and immunohistochemistry

Fixation was performed in St. Marie's solution (1% acetic acid and 96% ethanol for 24h, +4°C for immunohistochemical examination. Embryos were routinely processed and embedded in paraffin at 56°C. Serial sections were cut (5 μm), air dried for 24h, and deparafinized. For detection of PCNA mouse monoclonal antibody (M 0879 – DAKO). Negative controls were treated with an unspecific antibody (V 1617 mouse IgG_1, DAKO). Hydrogen peroxide block was applied for 5 min and slides were washed in buffer solution. As wash and buffer bath was used 0.05 Tris HCl pH 7.6 containing 0.3M NaCl and 0.1% Tween 20. Primary antibody (1:50) was applied for 30 min and washed. A sensitive labeled streptavidin-biotin kit (DAKO LSAB®2 Kit, HRP, Rat) was used for detection of primary antibody. Link antibody consisting of secondary bitinylated antimouse and anti-rabbit antibody was applied for 30 min, washed in buffer, streptavidin--peroxidase complex was applied for 30 min and washed, sections were incubated with substrate-chromogen solution containing diaminobenzydine (DAB) for 2 min and finally briefly counterstained with hematoxylin, washed with water, mounted with glycerol and PBS (1:1).

Stereological analysis

For the stereological analysis, randomly chosen paraffin blocks were used and cut extensively by a rotation microtome. After obtaining serial sections, the volume was estimated according to the Cavalieri method. For the evaluation of immunohistochemistry (IHC)-positive cells, five consecutive sections were taken in a random fashion from each series. The stereological analysis was performed on the first and the fifth section applying a physical dissector principle. Stereological analysis was carried out using a Nikon Alphaphot binocular microscope (Nikon, Vienna, Austria). Weibel multipurpose test system (42 points) was applied at 400x magnification. The numerical density of IHC-positive cells was determined according to the point counting method.

Statistical analysis.

The assessed stereological data for PCNA-positive cells were evaluated by descriptive statistics and correlation matrix (Biostatistical package microstat, School of Public Health, Medical School, University of Zagreb). For statistical evaluation different medium on growth Student's test and X^2 test were used.

Results

Expression of the PCNA in foetuses cultivated with the fibroblast growth factor

First the experiments were done to investigate expression of the proliferating cell nuclear antigen (PCNA) in foetuses cultivated with the fibroblast growth factor. Experimental group foetuses were cultivated in a minimum essential medium (MEM) with 50% rat serum supplemented with 100 ng/ml FGF, whereas foetuses culti-

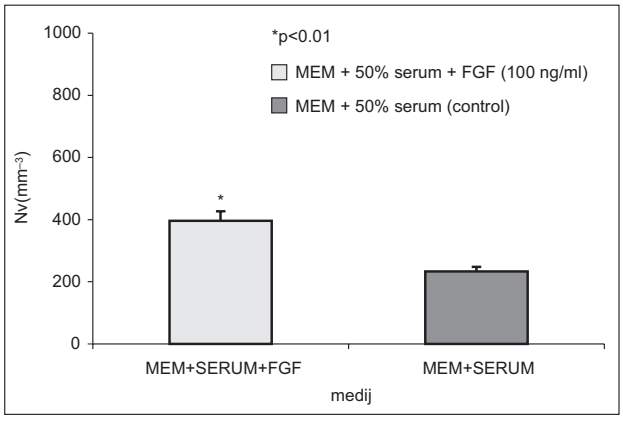


Fig. 1. Expression of PCNA in the rat embryos (9 days old) in neural tissue differentiated after 14 days of culture in medium with serum and FGF.

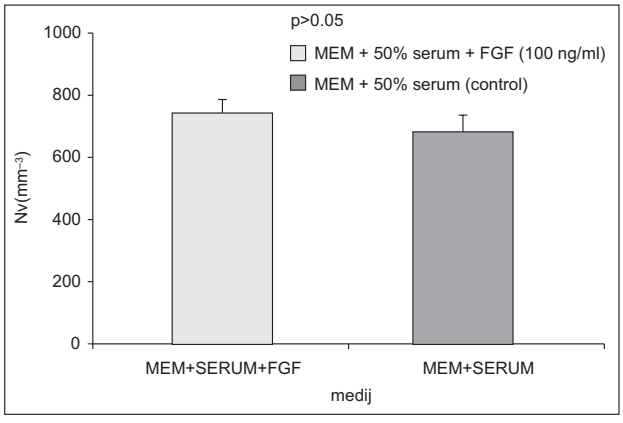


Fig. 2. Expression of PCNA in the rat embryos (9 days old) in cartilage tissue differentiated after 14 days of culture in medium with serum and FGF.

vated in MEM with only 50% rat serum served as a control. After 14 cultivation days a clear intranuclear signal was established in all the tissues differentiated in foetuses. Further analyses of expression of PCNA were applied on cartilage tissue and nerve tissue. PCNA signal was present in most of the cartilage cells which shows that these cells have mainly kept their proliferating ability. I difference to cartilage cells, expression of PCNA in the nerve tissue was detected in fewer cell number, which shows that these cells have mainly lost their proliferating ability, probably because they have started to differentiate terminally. Stereological quantitative analysis of expression of PCNA signal in both nerve tissue and cartilage, which was applied according to numerical density value analysis (Nv) showed that the nervel tissue growth differentiated in foetuses cultivated in a medium supplemented with FGF was statistically much higher ($p<0.01$) than in foetuses cultivated in control medium (Figure 1), whereas the cartilage growth was almost equal ($p>0.05$) in both experimental and control group foetuses (Figure 2).

Growth of whole foetuses cultivated with the fibroblast growth factor

Latter experiments have shown that there is a tissue specific difference in expressionof PCNA. So, under the influence of FGF, a difference in proliterating ability of some foetus tissues appears. To establish the FGF influence on foetus growth a classic method was applied in measuring whole foetus diameters. 8-and 9-day-old foetuses were measured during 14 culture days. Growth influenced by FGF in a medium with serum, as well as in serum free media (pure MEM and MEM supplemented with transferin) was investigated.

a) 8-day-old foetus growth in a medium with serum and FGF. Experimental group of 8-day-old foetuses was grown in MEM with both rat serum (1:1) and FGF (100 ng/ml), and the control group was grown in MEM with only rat serum (1:1). Larger and smaller diameters were measured couple of times during 14 days of culture. Foetus growth curves (Figure 3) show higher growth in a control medium than in a medium supplemented with

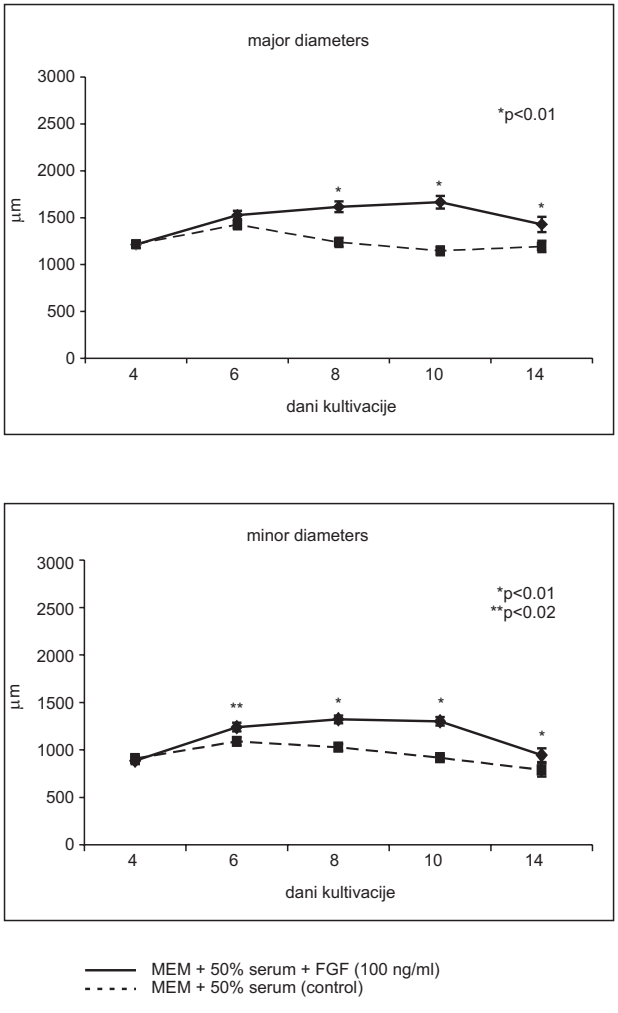


Fig. 3. Diameters of 8 days old rat embryos cultivated in medium with serum and FGF addition during 14 days.

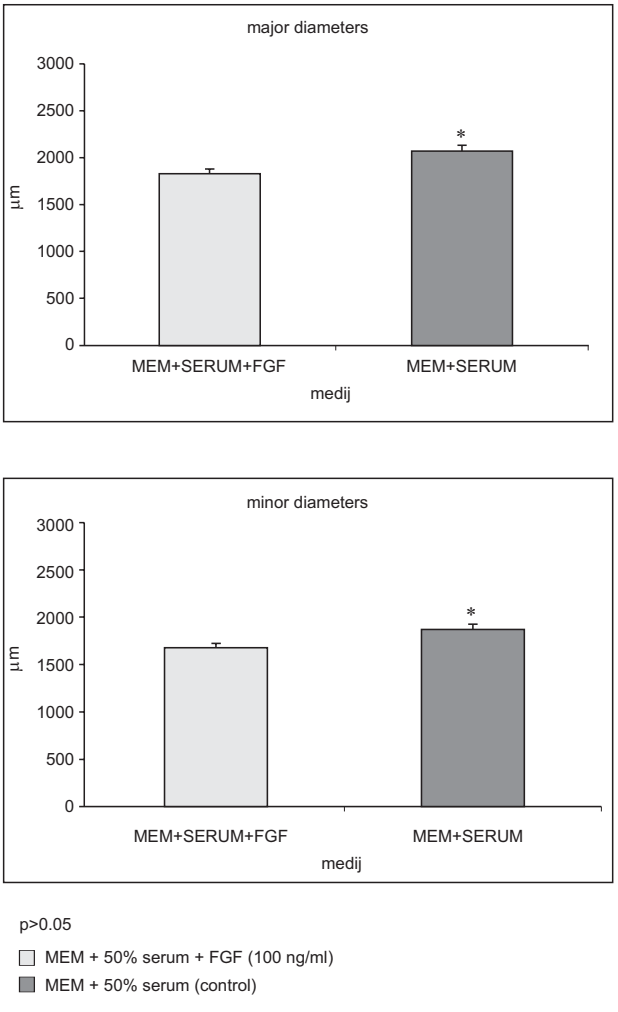


Fig. 4. Diameters of 9 days old rat embryos cultivated in medium with serum and FGF. Measured on 7th day of culture.

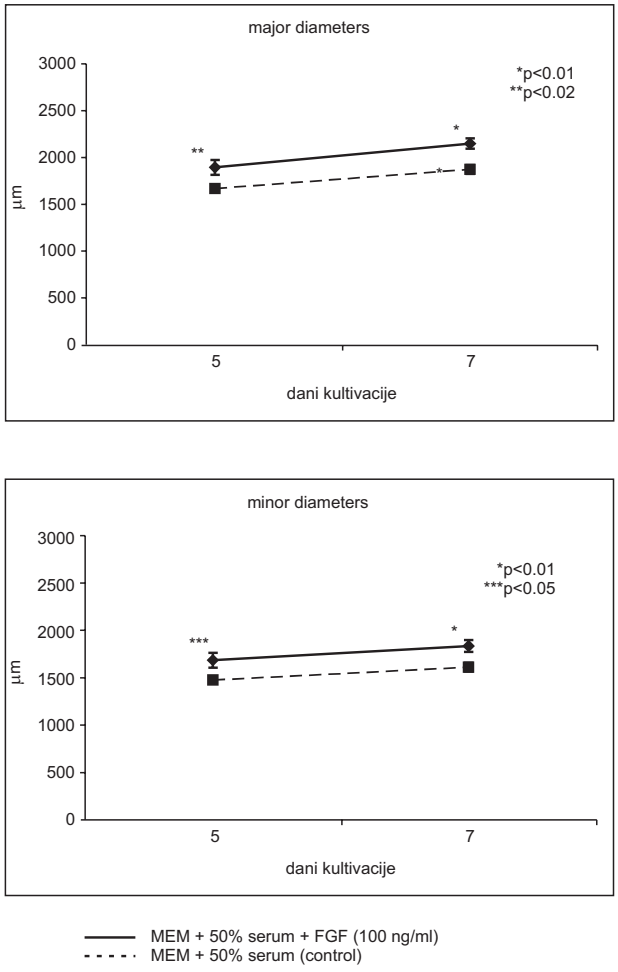


Fig. 5. Diameters of 9 days old rat embrxos cultivated in medium with serum and FGF addition. Measured on 5th and 7th day of culture.

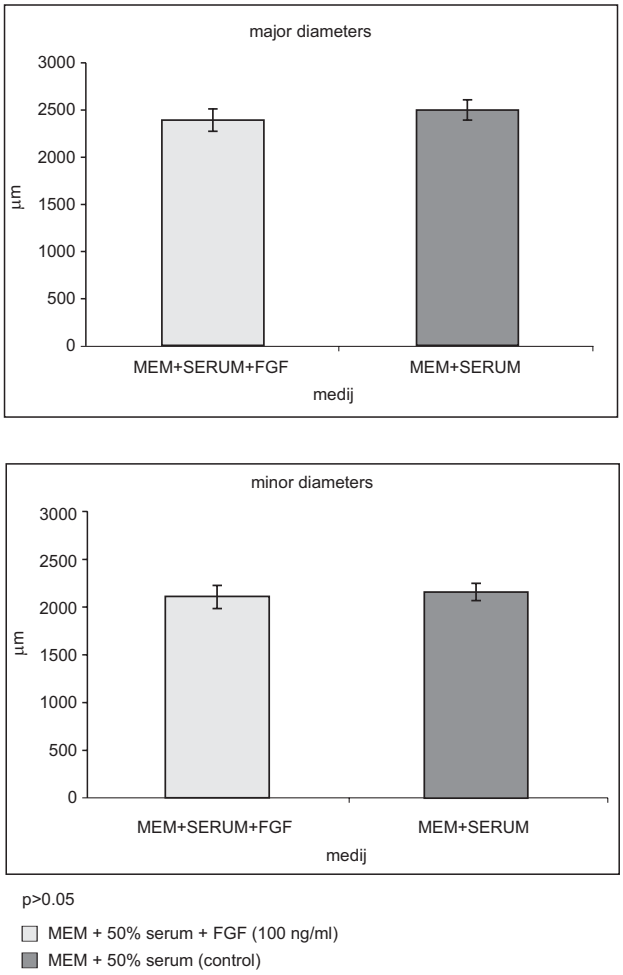
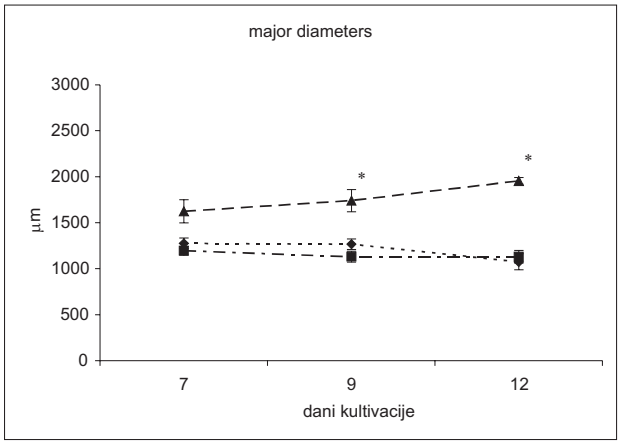


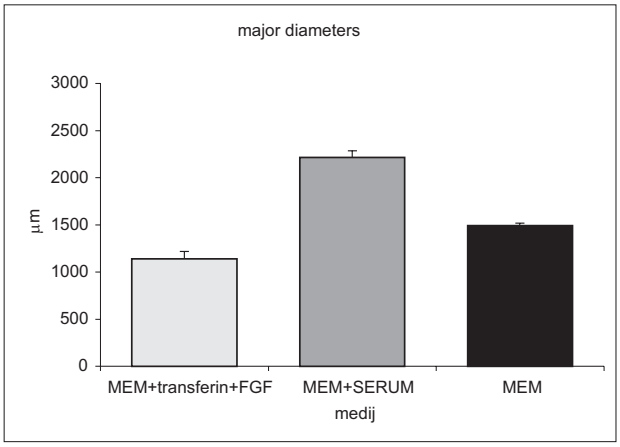
Fig. 6. Diameters of 9 days old rat embryos cultivated in medium with serum and FGF. FGF added after 7 days of culture. Measured on 14th day of culture.

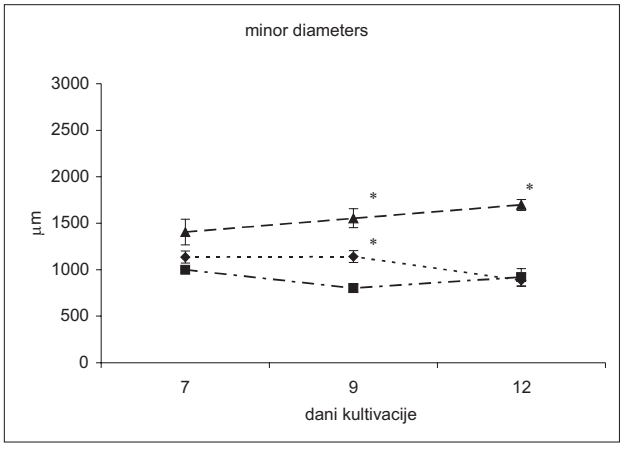
FGF. In a control the growth increases till culture day 10. Between days 10 and 14 the sudden growth fall occurs. In a medium supplemented with FGF the growth increases till culture day 6, and then decreases gradually. Comparisons have shown that the highest statistically significant difference ($p<0.01$) in foetus growth investigated media exists in the second week of culture.

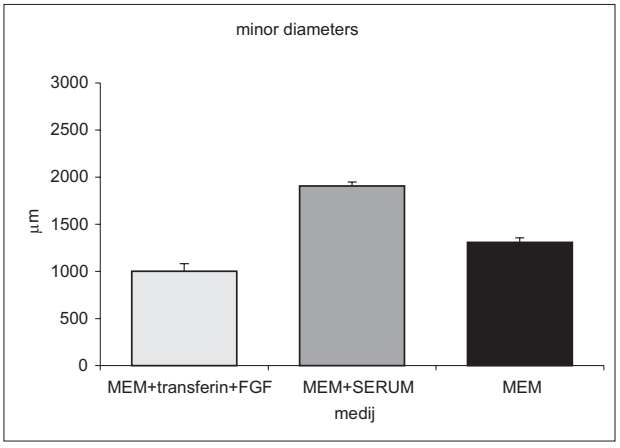
b) 9-day-old foetus growth in a medium with serum and FGF. In the following three experiments done on 9-day-old foetuses the control was MEM with rat serum, and the experimental MEM with rat serum supplemented with FGF (100 ng/ml). In the first experiment FGF was supplemented at the very beginning of the culture, in the period when foetuses grow intensively. The foetuses were cultivated for 7 days and the measuring of largerer and smaller diameters was done in both control and experimental group. The growth in a control group was statisticaly pretty higher ($p<0.01$) than in experimental group (Figure 4). In the second experiment the same media were used and the growth in both control and experimental group was measured on days 5 and 7. Also in this experiment the growth in control was statisticaly pretty higher ($p<0.05$; $p<0.02$; $p<0.01$) than in experimental group (Figure 5). In the third experiment foetuses were cultivated for 14 days and the FGF was supplemented after 7 days, that is the period when the foetus growth falls. The growth was measured on day 14 in both control and experimental group. This experiment's results have shown no difference in growth between the experimental and control groups ($p>0.05$) (Figure 6). The experiments have shown that the FGF lowers the growth of rat foetuses cultivated *in vitro* if it is applied during the first culture week.

c) 9-day-old foetus growth in MEM with FGF. This experiment's aim was to establish the FGF's influence on the growth of foetuses cultivated in pure MEM, free of any other protein supplements. Experimental group was cultivated in a serum free medium supplemented with FGF (100 ng/ml). One control group was cultivated in a medium with serum (both MEM and 50% rat serum), whereas the other control group was cultivated in a se-









*p<0.01

MEM + FGF (100 ng/ml)
MEM + 50% seruma (control)
MEM (control)

Fig. 7. Diameters of 9 days old rat embryos cultivated with FGF in serum free medium.

*p<0.01

MEM + transferin (50 mg/ml) + FGF (100 ng/ml)
MEM + 50% serum (control)
MEM (control)

Fig. 8. Diameters of 9 days old rat embryos cultivated with FGF in serum free medium with transferin. Measured on 6th day of culture.

rum- and protein free MEM. Measurings of foetus diameters were done till culture day 12. The highest growth was noticed in a medium with serum, a bit lower growth appeared in MEM, and the lowest growth was in a medium with FGF (Figure 7). This can best be seen on the curve showing smaller diameters measured on culture day 9, whereas the last measuring done on culture day 12 shows equalization of foetus diameters values, cultivated in both MEM and FGF.

d) 9-day-old foetuses growth in MEM supplemented with transferin and FGF. This experiment's aim was to establish how FGF changes the growth of whole foetuses in a medium with transferin. A serum free medium was applied because the previous research has shown the better foetus growth in a serum free medium than in pure MEM (Bulić-Jakuš, 1990). Experimental group foetuses were cultivated in MEM supplemented with transferin (50µg/ml) and FGF (100ng/ml). One foetus control group was cultivated in a medium with serum (MEM and 50% rat serum), and the other control group was cultivated in pure MEM. The growth was measured on culture day 6. Comparison of the average foetus diameter rates has shown the highest growth in a medium with serum, a bit lower was the growth in MEM, and the lowest growth was noticed in a medium with FGF. It has also been shown that all the differences among the compared media are statisticaly significant ($p<0.01$) (Figure 8).

e) 9-day-old foetus growth in MEM with transferin and the lower FGF concentration. In the next experiment the FGF concentration was lowered due to possibility that the 100 ng/ml concentration used in previous experiments was too high to motivate the growth of whole foetuses. One experimental group was cultivated in MEM with transferin (50 µg/ml) and FGF (12,5 ng/ml), the other group was cultivated in MEM with only transferin (50 µg/ml). The control group was cultivated in pure MEM. Foetuses were cultivated for 14 days, the mea-

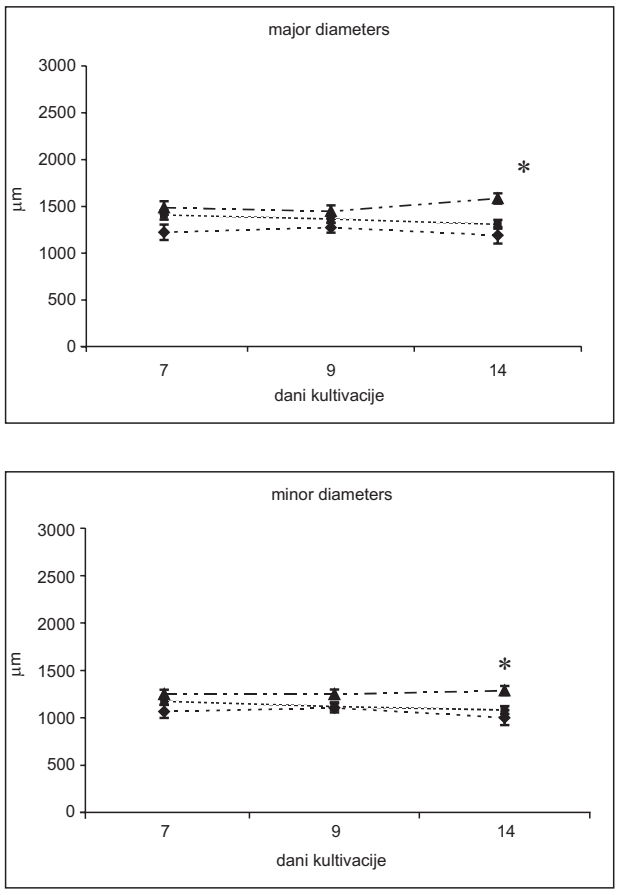


Fig. 9. Diameters of 9 days old rat embryos cultivated with FGF in serum free medium with transferin.

surings were done on culture days 7, 9 and 14. The highest growth was in a medium with both transferin and FGF, a bit lower growth appeared in a medium with only transferin, and the lowest growth was in pure MEM (Figure 9). Statisticaly significant difference in diameter size ($p<0.01$) among foetuses cultivated in a medium with transferin and those cultivated in a medium with both transferin and FGF was established when measuring on day 14, for both larger and smaller diameters. This shows that the lower FGF concentration improved the foetus growth because in all previus experiments the growth was lower in a medium with FGF than in a control medium.

Discussion

The experimental *in vitro* culture model for mammal foetus growth cultivation has ben used for researching the expression of gen for the proliferating antigen. During 14 days the PCNA expression has been proved in postimplant rat foetuses cultivated in an *in vitro* culture model even in the serum- and protein – free foetuses. The growth could not be established in a protein-free medium when applying a classic method of measuring whole foetus diameters with the help of ocular micrometer[14], but it has been proved that those foetuses transplanted *in vivo* continue their growth and inside of them derivates of the basic tissues appear which then differentiate *in vitro*[15]. In this research paper based on the expression of PCNA, the endogen proliferation mark[16], a direct proof has been detected to show that there is a preserved proliferating ability in those foetuses.

Detecting the proliferating ability in foetuses cultivated *in vitro* made it possible for us to study the single tissue proliferating ability under the influence of fibroblast growth factor. The fibroblast growth factor family (FGF) belongs to the group of parachryne growth and differentiation factors[17]. Numerous testings in embriology, cell biology, molecular and clinical biology have pointed out to the role and the importance of FGF in the growth processes and differentiation[18,19]. The study of cartilage cells as well as nerve tissue cells is considerd to be one of the mostly applied methods when studying the FGF's influence on the cell proliferation and differentiation[20], that is the reason why the expression of PCNA has specially been analysed in those tissues.

Although it has been known that FGF has an important role in maturing of the hondrocytes, it is still just a little known about the mechanism which effects the proliferation and differentiation of these cells[21]. FGF2 stimulates the hondrocytes proliferation but at the same time it blocks their differentiation. FGF9 also stimulates proliferation but lesser than FGF2, and it inhibits the terminal differentiation of the hondrocytes[22]. Interactions between family members of fibroblast growth factors and their receptors have an important role in interfering in intercell interactions during the embriogenesis. Signalization over receptor3 for the fibroblast growth factor (Fgfr3) can either stimulate or inhibit the hondrocyte proliferation depending on the foetus growth period[23,24]. FGF also effects the intercell substance in the way that it stimulates the proteoglycane synthesis[25]. In this research the presence of PCNA signal has been established in the majority of cartilage cells which shows that they keep their proliferating ability even after the 14 days of cultivation in the *in vitro* culture model. Stereological analysis has been done but it showed no significantly important difference in growth process between the cartilage differentiated in a control medium and in a medium supplemented with FGF, probably due to the cartilage's high proliferating ability at that moment.

Fibroblast growth factor is also included in neural induction[26,27]. The experiments of cultivating the whole postimplant foetuses are suitable for researching the various aspects of neurulation among mammals[28]. The survival of each neuron during the embryonal growth process has been defined by interaction of many factors, including the FGF[29]. FGF supports the survival of neurons in a rat's brain[30,31], the same as the survival of neurons in a chicken's retina[32].This research paper has

proved that FGF increases the number of nerve tissue cells that kept their proliferation ability, which can be seen according to the increased PCNA expression. This result could mean that FGF also supports the survival of nerve tissue cells in the *in vitro* culture model. Measurings of whole foetus diameters have shown that FGF, if applied in the first culture period, during the intensive foetus growth, lowers the growth no matter what the foetus age at the moment of culturing is and no matter the medium (with or without a serum). If FGF is applied in the second culture period, it lowers the gowth of 8--day-old foetuses, however the 9-day-old foetuses growth decrease has not been that suggestive. Accordingly, it seems that younger foetuses in the *in vitro* culture model are more sensible to the negativeFGF influence on the growth in the second culture period.

When FGF was applied in a lower concentration the growth of whole foetuses cultivated in the *in vitro* culture model improved, which shows that FGF influence on growth depends on concentration. This result affirms the importance of concentration of growth factor and differentiation in the growth process regulation[33]. The combination of classic method of measuring whole foetuses diameters and the analysis of proliferating antigen expression at the single cell level appears to be suitable for precise quantitative studies of growth processes in the cultivated foetuses.

In vitro culture model applied in this research is the only one that kind which can studies direct influence of various outside factors on postimplant rat foetuses in chemically defined media. Such a model made it possible to study growth factors, differentiation, morfogens and teratogenic factors more precisely[34] without the obstructing serum effect that is unpredictable and often variable[35].

So far the influence of the outside factors on the proliferating ability in this model could have been observed only by measuring whole foetuses growth[36] but, it has now been seen that by immunohistochemical detection of proliferating antigen expression a difference can be determined in effecting the proliferating ability inside a single tissue. This will enable a more precise analysis of various positive and negative factors influence on the growth of postimplant foetuses in the *in vitro* culture model.

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UTJECAJ FAKTORA RASTA FIBROBLASTA (FGF) NA RAST I DIFERENCIJACIJU ŠTAKORSKIH ZAMETAKA KULTIVIRANIH U KULTURI *IN VITRO*

S A Ž E T A K

Istraživan je utjecaj faktora rasta fibroblasta (FGF) na rast i diferencijaciju štakorskih zametaka starih 8 i 9 dana, kultiviranih u kulturi *in vitro*. Proliferacija je praćena analizom ekspresije nuklearnog antigena proliferirajućih stanica (PCNA). Utvrđeno je da primjena FGF-a u prvom dijelu kulture smanjuje rast bez obzira na starost zametaka u trenutku nasađivanja i bez obzira na to da li je medij sa ili bez seruma. Ako se FGF primjeni u drugom dijelu kulture, također smanjuje rast, ali su mlađi zameci u kulturi *in vitro* osjetljiviji na njegovo negativno djelovanje. Kada je FGF primijenjen u nižoj koncentraciji poboljšao se rast čitavih zametaka u kulturi *in vitro*, što pokazuje da je njegovo djelovanje na rast ovisno o koncentraciji. Provedene stereološke analize su pokazale da, u kulturi *in vitro*, FGF ne utječe na proliferaciju tkiva hrskavice, ali podržava preživljenje stanica živčanog tkiva. Pokazalo se da se kvantitativna istraživanja procesa rasta u kultiviranim zamecima mogu precizno provesti kombiniranjem klasične metode mjerenja promjera čitavih zametaka s analizom ekspresije proliferacijskog antigena.