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APPLICATION OF ANTERIOR PITUITARY CELL CULTURE IN TOXICOLOGICAL RESEARCH

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High density plating procedure was used to evaluate the effect of atrazine on anterior pituitary cells of rats in monolayer culture. Collagenase-dispersed pituitary cells plated in suspension with medium-199 and 10% foetal calf serum attached quantitatively to plastic surfaces within 24 hours. Electron microscopy showed subpopulations of different cell types. After prolonged cultivation, most cells established small colonies with extensive contacts among them. Cell-to-cell formation of aggregates was significant and the colonies manifested morphological changes. The cells retained their enzymatic activity, converting testosterone into 5α-dihydrotestosterone by enzyme 5α-reductase. Immunohistochemical techniques facilitated differentiation of gonadotrophs producing follicle stimulating hormone (FSH) and luteinising hormone (LH). Atrazine in concentrations of 5 to 50 μg/ml of medium was associated with a significant reduction in the number of viable cells within 72 hours. The results suggest that the pituitary cell culture may prove useful in toxicological testing of various toxic compounds and reduce or replace *in vivo* animal experiments.

Key words:
atrazine, cell viability, experimental animals, gonadotrophs, *in vitro* system, monolayer culture, xenobiotics

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine), a selective *s*-triazine herbicide, has been widely used to inhibit the growth of broadleaf and grassy weeds harming corn, sorghum and other crops, as well as plants used in reforestation. The extensive use of atrazine and its 18-month degradation in nature makes possible its presence in raw and industrial food as well as in agricultural watersheds (1–4). The daily intake of subtoxic doses of pesticides such as atrazine can induce physiological changes without visible signs of intoxication. The chemical contamination of the environment presents a risk not only for the current population and to foetuses, but also for the future generations through possible mutations affecting the reproductive system (5–7). Some chemicals directly affect conceptus, while others like atrazine alter the reproductive function at the neuroendocrine level and/or in target organs such as hypothalamus (8, 9), anterior pituitary (8–10), prostate (11–13), and uterus (14).

The pituitary gland as the main gland of the neuroendocrine system plays a key role in the maintenance of homeostasis. There are at least three levels of feedback control in the hypothalamus and the pituitary gland at the neuroendocrine level: 1) long feedback systems, where hormones produced in the peripheral target glands provide inhibiting or activating messages; 2) short feedback systems, where hormones synthesised in the anterior pituitary gland are the controlling signals; and 3) ultrashort feedback systems, where the releasing factors directly influence the rate of hormone production (15, 16). The pituitary gland holds the major position in these complex gonadotrophic feedback mechanisms; any change in this gland will disturb the feedback mechanism.

Drug toxicity testing *in vivo* at the neuroendocrine level is complex and difficult. However, primary culture of pituitary cells allows complete isolation of the pituitary gland from the neural and humoral influence. Furthermore, it allows application of xenobiotics under controlled *in vitro* conditions.

In our study, we sought to produce a monolayer primary pituitary cell culture in favourable conditions so as to investigate the possibility of its application in toxicity testing. We applied a high density plating procedure to speed up the cell attachment and enhance the opportunity for cell-to-cell contact among neighbouring pituitary cells. The initial 3-day culture applied in our experiment corroborated the hypothesis of *Kniewald and co-workers* (17) that pituitary cells form direct intercellular associations between different cell types during *in vitro* cell culture. Atrazine was added to the pituitary cell culture in different concentrations in order to evaluate its toxicity by determining the density of viable cells.

The objective of this study was to find out whether the primary pituitary cell culture could be successfully introduced in the testing of potentially toxic compounds such as atrazine, with the ultimate goal to reduce or replace the complex and very expensive *in vivo* experiments.

MATERIALS AND METHODS

Animals

The Fisher strain male rats (90 days old) were obtained from the Animal Unit of the Faculty of Medicine, University of Zagreb, Zagreb, Croatia. The animals were kept in standard cages for rodents (4 to 5 animals in each) in a room with controlled temperature (23±2 °C) and relative humidity (55%). The light and dark cycles alternated every 12 hours. The animals had been given standard pelleted laboratory feed (PLIVA d.d. Zagreb Pharmaceutical Co., Zagreb, Croatia) and tap water *ad libitum.*

Forty-eight hours before experiments, the rats were castrated by the scrotal route under ether anaesthesia. The intact rats and the castrated rats were decapitated under light ether anaesthesia. The pituitary glands were removed immediately and washed from blood traces in Ca^{2+} and Mg^{2+} free Hanks balanced salt solution (CMF-HBSS) buffered with molar N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid (HEPES), and were rinsed thereafter in the same buffer.

Cell dispersion and culturing

Pituitary cells were dispersed using a modified method of *Kniewald and co-workers* (17). All procedures were carried out in a laminar-flow hood (Gelaire, Twin 30). Pituitary tissue was aseptically trimmed from adhering tissue residues, transferred to a sterile conical tube, and washed four times with 5 ml of CMF-HBSS buffer (pH 7.6–7.8). The medium was discarded and the glands were cut into 0.5 mm blocks with a sterile razor blade. The media and the tissue blocks were again transferred to conical tubes and centrifuged for 5 minutes at 700 x *g*. The supernatant was removed and the tissue was resuspended in dissociation medium [0.1% collagenase in MF-HBSS buffer (Mg^{2+} free Hanks balanced salt solution, with 15 μmol calcium and 3% bovine serum albumin) and 25 mmol HEPES pH 7.6] after modification of the method by *Wilfinger and co-workers* (18). The medium and the tissue were placed into a stirring flask with a mixture of 95% air and 5% CO_2, incubated at 37 °C, and stirred under controlled conditions for up to 90 minutes. Every 30 minutes of incubation, individual cells were separated from fragments by gentle trituration through a siliconised pipette. After a full-time incubation, the cell suspension was centrifuged for 5 minutes (700 x *g*). The supernatant was discarded and the cells resuspended in a 0.25% pancreatin in CMF-HBSS buffer, returned into the stirring flask and stirred for additional 15 minutes. The dispersed pituitary cells were removed from the stirring flask and transferred to a sterile centrifuge tube for a 5-minute spin (700 x *g*). The supernatant was discarded and the cells were resuspended in M-199, shaken, and filtered through a nylon mesh (160 μm) into a sterile flask. The medium volume was adjusted to achieve an initial cell suspension of $5x10^5$ cells/ml. After four washes, an aliquot was taken for the cell count with haemocytometer (0.4% trypan-blue stain) containing 16 small blocks of one million cells per millilitre. The cells were plated into sterile tissue culture plates with 24 wells and cultured in M-199 with the addition of a 10% foetal calf serum (FCS) in a CO_2 automatic incubator at 37 °C. All chemicals for the experiments were supplied by Sigma, Cheminst GmbH (Diesenhofen, Germany). All media, the serum, and solutions used for culturing were filtered through a 0.22 μm membrane filter (GS, Millipore filter). All glassware and equipment were sterilised by autoclaving.

Incubation procedure for testosterone metabolism

(4-^{14}C)-Testosterone (specific activity 2.15 Gbq/mmol), supplied by Radiochemical Centre Amersham (Bucks, UK) was purified by thin layer chromatography (TLC) before use. The pituitary cells ($3x10^5$ cells/ml of medium) were left 24 hours in culture with M-199 plus 10% FCS and then washed twice with M-199 without serum, before adding the labelled testosterone. ^{14}C-Testosterone (166 ng/ml of M-199) was incubated for 24 hours with isolated pituitary cells at 37 °C in a CO_2 automatic incubator (Flow Laboratories, USA). The testosterone metabolites were identified according to the procedure described by *Kniewald and co-workers* (11).

Immunohistochemical staining of pituitary cells

Dispersed rat pituitary cells were cultured in a slide flask containing serum-supplemented medium (5x10^5 cells/ml of medium) for seven days before application of Rabbit Anti-Human Follicle Stimulating Hormone and Rabbit Anti-Human Luteinising Hormone (DAKO Corporation, USA). On the seventh day, the slide flasks were taken out of automatic incubator, opened, the medium discarded, and cells processed for immunohistochemistry, as previously reported (18). The cells left on slides were fixed for 30 minutes in Bouin (mixture of 36% formaldehyde, glacial acetic acid, and picric acid). The treatment with normal swine serum (3% serum in PBS) before administration of primary antibody was necessary to reduce background staining. Immunohistochemical staining followed manufacturer's instructions. All incubations were performed at room temperature as follows: a) primary antibody was incubated for two hours; b) link antibody was incubated for 20 minutes; c) streptavidin peroxidase was incubated for 30 minutes. The application of diaminobenzidine-H_2O_2 (DAB-H_2O_2) for five minutes allowed visualisation of the reaction (19). Washed with PBS, the cells were incubated in hemalaun and eosin for few minutes. Slides were routinely processed through graded alcohol.

Electron microscopy of pituitary cells

After the long-term cultivation (72 hrs), the cultured pituitary cells were washed twice with M-199 to remove all traces of serum for electron microscopy. The pituitary cells were detached with trypsin from the surface of the plates. Followed the fixation in a 1% glutaraldehyde for 25–30 minutes at 4 °C and postfixation in a 2% osmium tetroxide in 0.05 mol cacodylate buffer (pH 7.2). The cells were then dehydrated and embedded in Polybed 812/Araldite capsules. The sections were stained with uranyl acetate and lead citrate and examined using a Zeiss EM 902 A electron microscope, at the Institute »Ruđer Bošković« in Zagreb.

Incubation procedure for atrazine toxicity

Due to its liposolubility, atrazine solution was prepared in absolute ethanol. In order to determine influence of ethanol on pituitary cell monolayer development, different volumes (10–50 μl/ml M-199) of ethanol had previously been tested. The volume of 10 μl of ethanol showed no significant effect on the cell monolayer development. Therefore, 10 μl of atrazine solutions in concentrations of 5, 25, 37.5, or 50 μg/ml of medium were added to each well. One milliliter of pituitary cells containing 3x10^5 cells was added to each well. The cultivated pituitary cells were in contact with different concentrations of atrazine for up to 72 hours. Atrazine inhibition was assessed by measuring the density of viable cells in 24-, 48-, and 72-hour cultures. The percentage of viable cells was calculated in relation to the values measured in the control culture with ethanol.

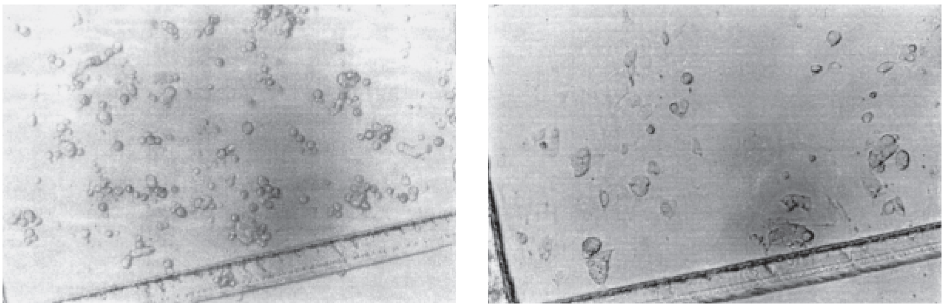
Statistics

The results of replicate experiments are presented as means of viable cell density and standard errors. Significant differences between values in cultures with atrazine and the control culture with ethanol were identified by Student's *t*-test.

RESULTS AND DISCUSSION

The functional significance of the cellular organisation of the pituitary gland and the role of intercellular communications among homologous and heterologous cells have not yet been understood fully. Pituitary cells are anchorage-dependent and must attach to a suitable surface (substratum) before they can grow. Enhancement of cell attachment is achieved by initially plating the cells in very small volumes such as 1 ml, and allowing them to settle for at least 8 hours. The dispersed pituitary cells in the stationary monolayer cultures undergo significant morphological reorganisation and aggregation.

Figure 1A shows freshly dispersed cells. They are round or spherical and are routinely found randomly distributed over the surface of a plate or a well. Most cells lay within a distance of 10 μm from a neighbouring cell when the cultures are plated at a density of $3x10^5$ cells/ml. After a 24-hour culture period, the cells re-established small colonies on the surface of the plate, established contact with at least one neighbouring cell, and remained round. They did not start to spread over the plate surface, but they started to flatten and extended cellular appendages. Forty-eight hours later the cells appeared more planar and the formation of aggregates was evident.



A B

Figure 1 *A – Dispersed pituitary cells in concentration of $3x10^5$/ml (magnification 250x); B – 72 hrs after the cells established contacts with neighbouring cells (magnification 360x).*

After 72 hours, the cells spread even more, and the cell-to-cell contact appeared abundant (Figure 1B). Most cells spread over the plate surface. They flattened and established contacts with neighbouring cells, enabling intercellular communication and the exchange of material.

When freshly dispersed cells suspensions were plated at an alkaline pH 7.6 in a medium containing low extracellular calcium concentrations, they formed a layer of single cells and attached to the surface of the plate 24 hours after sedimentation. The quantitative efficiency of the cell attachment to the surface of the plate (80%) could be achieved after a 24-hour incubation period. In order to reduce the time required for the cells to attach, we used 15 μmol calcium in resuspension and plating medium.

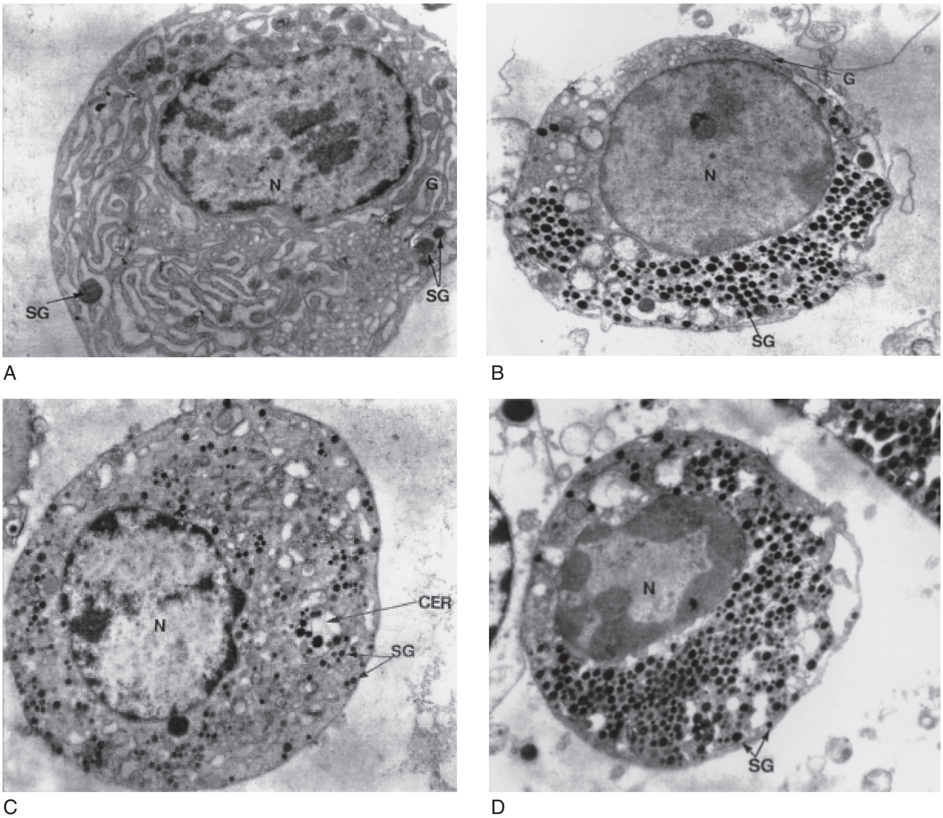


Figure 2 *Electron microscopy of pituitary cell culture suspension. A – Mammotrophic cell; B – Somatotrophic cell; C – Gonadotrophic cell synthesising FSH; D – Gonadotrophic cell synthesising LH (magnification 7500x). N – Nucleus; G – Golgi apparatus; SG – Secretory granules; CER – Endoplasmatic reticulum.*

Cell clumping was eliminated and the individual cells rapidly sedimented to the surface of the dish. The cell attachment was also enhanced with plating at an alkaline pH (7.6–7.8). The cell suspensions were plated at optimal density of $3x10^5$ cells/ml. Individual cells were usually within a 10 μm distance from the neighbouring cells. Our results suggest that cell-to-cell contact was required for optimal cell function in the culture. When cells were plated at low densities, the contact between individual cells was poor because of the greater distance among neighbouring cells. Overcrowding, however, also produces an inhibitory effect, as it results in the accumulation of metabolic wastes, depletion of nutrients, and change in the pH of the medium.

The anterior pituitary gland of rats contains six major hormone-producing cell types. Electron microscopy of primary pituitary cell culture confirmed four of them (Fig. 2 A, B, C, and D). Magnified up to 7500 times, the pituitary cell suspension showed several different cell types. The oval or spindle-shaped cell containing large

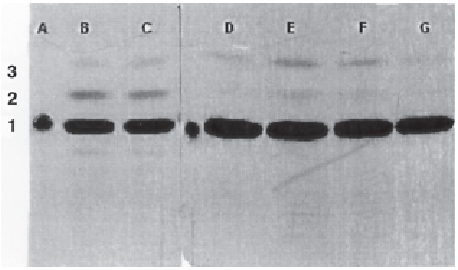
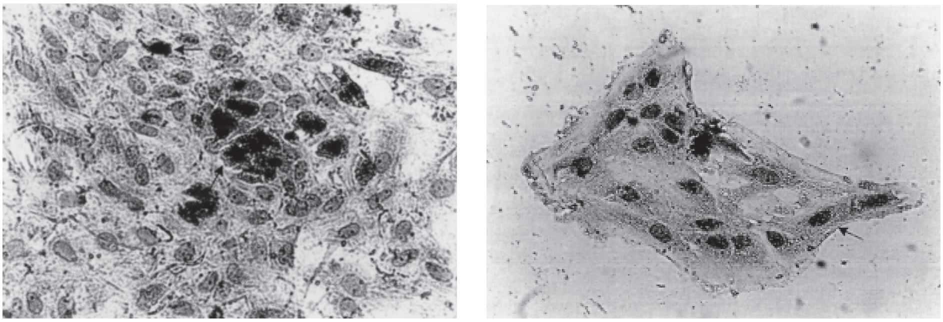


Figure 3 *Autoradiograph of (4-^{14}C)-testosterone metabolites after a 3-hour incubation of rat pituitary tissue and after a 24-hour incubation of pituitary cell culture: 1 – testosterone; 2 – 5α-DHT; 3 – androstenedione. A – (4-^{14}C)-testosterone, marker; B – pituitary tissue; C – pituitary tissue of rats 48 hrs after castration; D and G – pituitary cell culture; E and F – pituitary cell culture of rats 48 hrs after castration.*

secretory granules (SG) was identified as the mammotrophic cell (Fig. 2A). Golgi apparatus (G) is made of large cisternae and many smooth bubbles. Secretory granules (SG) are round or oval, and they could be found among Golgi apparatus and endoplasmic reticulum. The cytoplasm contains ribosomes, mitochondria, and lysosomes. Figure 2B shows the oval somatotrophic cells which synthesise the somatotrophic hormone, and contain many secretory granules (SG) occupying most of the intracellular space. The nucleus (N) is placed in the centre of the cell, and there is a big Golgi apparatus (G) next to it. The unoccupied surface area between the cell colonies is slowly filled with fibroblasts and endothelial cells. Gonadotrophic cells are more round and appear in two types: the first produce a hormone that stimulates the follicles (FSH) and the second produce luteinising hormone (LH). The gonadotroph which synthesises FSH (Fig. 2C) is big and round. Beside secretory granules, its cytoplasm contains mitochondria, cisternae of endoplasmic reticulum (CER), and a few ribosomes. Figure 2D shows the gonadotroph which synthesises LH. Like the FSH-synthesising gonadotroph, it is round but the Golgi apparatus is smaller and the nucleus (N) is either placed in the middle of the cell or is eccentric. Its secretory granules (SG) converge much more to one side of the cell than those in the FSH-synthesising gonadotroph.

In order to trigger the response of their target tissue, some hormones transform into »active« metabolites. Testosterone is converted into its »active« metabolite 5α-dihydrotestosterone (DHT) by the anterior pituitary gland, as well as by two peripheral androgen-dependent structures: prostate and seminal vesicles (20). In our experiment, the metabolism of (4-^{14}C)-testosterone occurred after it was added to the pituitary cell culture (Fig. 3). The enzyme 5α-reductase involved in the conversion of testosterone into 5α-DHT was detected in isolated pituitary cells and in the tissue of the whole pituitary gland. The conversion of (4-^{14}C)-testosterone into 5α-DHT in the anterior pituitary tissue of 90-day-old male rats 48 hours after castration yielded 795 pg of 5α-DHT/mg of wet tissue weight (21). The wet weight of pituitary cells in each experiment was approximately 0.9 mg (3.3 ng/cell) for the applied amount of $3x10^5$ cells/ml of M-199. The pituitary cells of male rats in culture (Fig. 3) retained the conversion characteristics of androgen hormones. The conversion yielded 1227 pg of 5α-DHT.

The dark spots on autoradiograph were identical to (4-^{14}C)-testosterone (Fig. 3, spot 1) and its active metabolite 5α-DHT (Fig. 3, spot 2). These results show the capability of pituitary cells in culture to convert testosterone into 5α-DHT within 24 hrs after adding it to the well. The pituitary cells retained their enzymatic property of converting testosterone into 5α-DHT with 5α-reductase. The pituitary cells of castrated rats showed better testosterone conversion than those of control rats, which corroborates the previous findings for the anterior pituitary tissue of male rats (20). The conversion process was activated by the absence of endogenous testosterone through castration and the increase in the activity of the 5α-reductase was obvious.



A B

Figure 4 *Immunohistochemistry of different types of pituitary cells:*
A – follicle stimulating hormone secreting cells;
B – luteinising hormone secreting cells (magnification 400x).

Immunohistochemistry of cultured pituitary cells in monolayer (Figures 4A and 4B) was applied to separate different types of the pituitary cell population. Figure 4 shows FSH secreting cells stained with streptavidin peroxidase, while Figure 4B shows the homogenous population of LH secreting cells, both after 7 days of cultivation. The application of immunohistochemical techniques in the pituitary cell culture made it possible to differentiate cells for FSH and LH production. The same techniques can be applied for toxicity testing of other compounds with potential neurotoxic or reproductive effects on the pituitary level.

Figure 5 shows the effects of atrazine on the viability of pituitary cells of rats in the primary culture. The average density of viable cells after 24, 48, and 72 hours, calculated as percentages of control values with the addition of 10 μl ethanol, was 94%, 79%, and 65%, respectively. Regardless of the limited new biomass production within 72 hours, sensitivity to atrazine was obvious. Atrazine inhibitory effect was significant in all concentrations. At 5 μg/ml it was 31% ($P<0.01$). In general, the inhibitory effect grew with the increase in atrazine concentrations in the medium; at 25 μg/ml it was 35% ($P<0.01$), at 37.5 μg/ml it was 41% ($P<0.01$), and at 50 μg/ml it was 48% ($P<0.01$).

The 24-hour incubation showed that the inhibition with atrazine concentrations of 25, 37.5, and 50 μg/ml ranged from 17% to 24% ($P<0.05$), whereas the lowest atrazine concentration of 5 μg/ml produced no inhibitory effect. The inhibition with

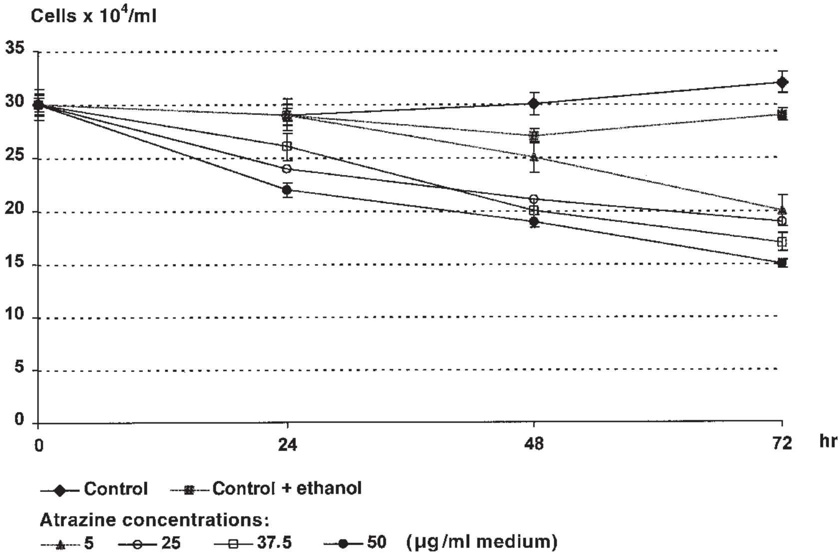


Figure 5 *The effect of different atrazine concentrations (µg/ml of medium) on the density of viable cells in the primary culture of rat pituitary cells.*

atrazine concentrations of 25, 37.5, and 50 µg/ml, found after 48 hours of incubation, ranged from 22% to 30% ($P<0.02$), whereas the inhibition withatrazione concentration of 5 µg/ml of medium reached only 8%.

CONCLUSION

Reproductive toxicology using the pituitary cell culture has only started to develop. The presented data show the inhibitory effect of atrazine on the pituitary cell biomass production within 72 hours of incubation by measuring the number of viable cells. On the basis of our results, it may be concluded that cultured pituitary cells can be successfully used to assess neurotoxic or reproductive toxic potentials of various compounds and may replace or reduce experiments on animals.

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Sažetak

PRIMJENA KULTURE STANICA PREDNJEG REŽNJA HIPOFIZE U TOKSIKOLOŠKIM ISTRAŽIVANJIMA

Atrazin, selektivni *s*-triazinski herbicid, danas se rabi u velikim količinama za zaštitu kukuruznih polja, a zbog svoje liposolubilnosti i perzistentnosti u okolišu ulazi u hranidbeni lanac. *In vitro* sustavi tkiva i organa sisavaca uz *in vivo* pokuse još i danas su pretežite tehnike u toksikologiji koje se pouzdano primjenjuju. Potrebe za smanjenjem broja pokusa na životinjama dovele su do intenzivna razvoja tehnika sa životinjskim stanicama, bilo da se radi o primarnim kulturama ili o staničnim linijama. Primarne kulture stanica središnjega živčanog sustava nisu do danas doživjele punu primjenu. Primjena životinjskih stanica mnogo je jeftinija tehnika koja omogućuje provođenje istodobnog ispitivanja na velikom broju uzoraka.

Cilj istraživanja bio je ispitati toksičnost atrazina u *in vitro* uvjetima na modelu kulture stanica hipofize, koje su odgovorne za normalno funkcioniranje reprodukcijskog procesa. U radu se dobrom izolacijskom metodom i visokim stupnjem uspješnog uspostavljanja kulture stanica, mogao pratiti utjecaj atrazina na razini staničnih promjena. Stanice raspršene kolagenazom u suspenziji s medijem 199 i 10%-tnim telećim serumom prihvatile su se za plastičnu podlogu unutar 24 sata. Elektronskom mikroskopijom nađeni su različiti tipovi stanica. Nakon produljene kultivacije, najveći broj stanica stvorio je male kolonije s uočljivim vezama između stanica. Stanice su pokazale dobru enzimsku aktivnost pri pretvorbi testosterona u aktivni metabolit 5α-dihidrotestosteron. Imunohistokemijskim metodama utvrđene su stanice koje sintetiziraju hormon što stimulira razvoj folikula (FSH) i hormon luteinizacije (LH). Atrazin je dodan kulturi stanica hipofize u koncentracijama 5–50 μg/ml medija koji je sadržavao $3x10^5$ stanica. Tijekom 24, 48 i 72 sata nađena su značajna smanjenja broja živih stanica, od 36 do 46%. Učinci su ovisili o dozi atrazina. Rezultati upućuju na to da se kulture stanica adenohipofize mogu uspješno rabiti u budućim istraživanjima toksičnosti različitih tvari radi smanjenja ili zamjene *in vivo* pokusa na životinjama.

Ključne riječi:
atrazin, gonadotropne stanice, *in vitro* sustav, jednoslojna kultura, ksenobiotici, pokusne životinje, vitalnost stanica

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