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# Influence of ghrelin on rat pituitary GH3 cell line proliferation

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## ABSTRACT

**Introduction.** Human ghrelin is the endogenous ligand of the growth hormone secretagogue receptor type 1a (GHSR1a). It is suggested that ghrelin is involved in pituitary adenomas pathogenesis. There are inconsistent data regarding the effect of ghrelin on cell proliferation. In this study the outcome of ghrelin in the rat pituitary adenoma GH3 cell line on morphology and proliferation ratio was evaluated. The ghrelin receptor (*Ghsr*) mRNA expression in GH3 cell line was established as well, because it was found that heterogeneous expression pattern characterized physiological and pathological conditions of tissues of different origin.

**Material and Methods.** Suitable experimental model pituitary tumor (rat GH3 cell line) was stimulated with ghrelin in the final concentrations  $10^{-12}$  M,  $10^{-9}$  M and  $10^{-6}$  M. Reverse transcription followed by real time polymerase chain reaction was used for ghrelin receptor gene transcript detection. The morphology as well as cell cycle of those cells were analyzed using Axio Vert.A1 Microscope (Zeiss) and BD FACSCalibur™ flow cytometer (Beckton Dickinson), respectively. The percentages of cells in the  $G_0/G_1$ , S,  $G_2/M$  cycle phases were evaluated using the ModFit™ software (Verity Software, Inc., USA).

**Results.** *Ghsr* mRNA presence was confirmed in GH3 cells. Ghrelin did not affect conspicuously GH3 cells morphology, however the ghrelin-induced proliferation index increase was caused by both decline of  $G_0/G_1$  phases cells count and increase those being in S+ $G_2/M$  ( $p < 0.05$ ).

**Conclusions.** In conclusion, this study indicates that ghrelin stimulates GH3 cells proliferation and may play role in pituitary tumorigenesis via an autocrine/paracrine pathway.

**Keywords:** ghrelin; ghrelin receptor; proliferation, pituitary adenoma.

## Introduction

The role of ghrelin (GHL) in pituitary tumorigenesis is unexplained so far. Expression of the receptor through which ghrelin mediate its effects was identified in different types of pituitary tumors, including the majority of somatotropinomas, in which the highest expression of growth hormone (GH) secretagogues receptor (GHSR) was detected [1]. There are

reports suggesting that ghrelin may be an antiproliferative factor. Its inhibitory impact on proliferation was confirmed in studies conducted on cancer cell lines of the thyroid gland, breast, pituitary and lungs [2]. Other studies suggest that it may also stimulate cells proliferation [3, 4].

GH3 cell line derived from rat's anterior pituitary tumor is an *in vitro* model to study pituitary adenoma

development. In the previous studies GH3 cells were also used as a suitable cell line model regarding human somatotropinoma. There are only few reports concerning the proliferative effect of ghrelin on somatotrophic cells of above mentioned cancer cell line [5–7]. Nanzer *et al.* found that both the acylated and non-acylated ghrelin stimulated proliferation of cells of GH3 cancer cell line [5]. Stevanovic *et al.* and Milosević *et al.* after administration of ghrelin into rats cerebral ventricles, found that the weight of the pituitary, the volume of both, GH-producing cells and their nuclei increased. This suggests enhanced potency of ghrelin, which may contribute to its transcriptional activity [6, 7].

In this study we examined the *Ghsr* gene expression in GH3 cell line, and whether ghrelin affects somatotroph pituitary rat adenoma GH3 cell line morphology and proliferation ratio.

## Material and Methods

### Cell culture and stimulation

All experiments were performed on rat pituitary adenoma GH<sub>3</sub> cell line obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Ham's F-10 medium (Cytogen, Germany) supplemented with 2.5% fetal bovine serum (FBS, Biowest, USA), 15% horse serum (Sigma Aldrich, USA), 100 µg/mL penicillin, 100 µg/mL streptomycin (Cytogen, Germany) and 2 mM L-glutamine (Cytogen, Germany). Cell line was maintained in aseptic conditions at 37°C, 5% CO<sub>2</sub> in a humidified incubator and confirmed free of mycoplasma contamination through regular testing (Mycoplasma PCR Test Kit, AppliChem, Germany). Cells were cultured until 90% confluence. At this point they were washed with phosphate buffered saline (PBS, Biowest, USA) and detached from culture dishes with 0.25% trypsin solution (Biowest, USA). After 3 minutes of incubation trypsin was removed, complete growth medium was added and resuspended cells were transferred into sterile 6-well plates at a density of 100,000 cells per well.

### *Ghsr* gene expression analysis

GH3 cells prepared as described before were used for *Ghsr* expression analysis using reverse transcription followed by real time polymerase chain reaction. This method was performed to analyze the expression in qualitative and not quantitative way, because of its sensitivity and specificity (provided with the TaqMan® probes).

### RNA isolation and reverse transcription

Total RNA was extracted from GH3 cells using ready-to-use RNA 3-zone reagent (Novazym, Poland) according to manufacturer's protocol with modification in RNA precipitation step performed in -80°C instead of room temperature. The quality of total RNA and its concentration were analyzed with the use of NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The integrity was evaluated by ribosomal RNA bands analysis after electrophoretic separation of 1µg RNA in 0.8% agarose gel in 1xFA buffer (20 mM 3-[N-morpholino]-propanesulfonic acid (MOPS) (free acid), 5 mM sodium acetate, 1 mM EDTA, pH 7.0, Sigma-Aldrich, USA) and presence of ethidium bromide and 0.8% paraformaldehyde (Avantor, Poland) providing denaturing conditions.

RNA was reversely transcribed to cDNA in three-step reaction conducted in accordance with Transcriptor Reverse Transcriptase manufacturer's protocol (Roche, Germany) in the total volume of 10 µL. In the first step mixture of: 5 mM oligo(d)<sub>10</sub> (Genomed, Poland), RNA (0.5 µg) and RNase-, DNase- and pyrogen-free water (Life Science) was denatured 10 min at 65°C. Subsequently, the samples were cooled on ice. In the second step of incubation 10U/µL ribonuclease inhibitor (RNasin, Roche), 10 U/µL of Transcriptor reverse transcriptase (Roche), 100 mM dNTPs (Novazym) and 1x reaction buffer (Roche) were added. Thermal profile was as follows: 10 minutes at 25°C (binding of primers to the template), 60 minutes at 55°C (cDNA synthesis step) and 5 minutes at 85°C (enzyme denaturation). Until real time PCR was performed cDNA was stored in -20°C.

### TaqMan® real time polymerase chain reaction

RNA expression pattern analysis was performed using the LightCycler 2.0 carousel-based system. Real time PCR for the Rat (*Rattus Norvegicus*) *Ghsr* (GeneBank: NM\_032075.3) was conducted with TaqMan® hydrolysis probes (Roche) and primers (Genomed, Poland) designed with the Universal ProbeLibrary assay design on-line software (Roche). Sense and antisense primers and the fluorescent probes numbers were: 5'-AG-GAAGCTATGGCGGAGAC-3' and 5'-GAAAGCAAACCA-CCACAGC-3', probe #112 (Roche cat. N° 04693469001). Rat ready to use *Actb* (β-actin) Reference Gene Assay (Roche assay N° 5046203001) was used as internal control and for relative concentration ratio evaluation. Reactions were performed in the total volume of 20 µL reagent mix containing: 1x LightCycler® FastStart™ TaqMan® Probe Master mix (Roche), 5 µL of cDNA, 0.5



mM of each primer (Genomed) and 0.1 mM hydrolysis probe (Roche).

5  $\mu$ l of qPCR product was analyzed and compared with the Nova 100 molecular mass marker (Novazym) after electrophoretic separation in 2% agarose gel (FMC BioProducts, Rockland, ME USA) containing 1x Tris/Boric Acid/EDTA (TBE) buffer (Bio-Rad) at presence of 500ng/ml ethidium bromide (Sigma-Aldrich).

The presence of TaqMan<sup>®</sup> hydrolysis probes ensured the specificity of the reaction. ZymoClean<sup>™</sup> gel DNA recovery kit (Zymo Research, USA) was used for PCR products gel recovery and purification followed by Sanger sequencing analysis (Genomed).

### Cell morphology

Cells morphology was evaluated using Axio Vert. A1 microscope (Zeiss, Germany) after 24 and 48 hours of incubation with different ghrelin concentrations:  $10^{-12}$  M,  $10^{-9}$  M and  $10^{-6}$  M.

### Cell cycle analysis

Cells were treated with ghrelin for cell cycle analysis in different final concentrations:  $10^{-12}$  M,  $10^{-9}$  M and  $10^{-6}$  M. All analyses were made in triplicates. Control contained non-stimulated cells. Cells prepared due to this procedure were used for further investigations.

After 24 and 48 hours of incubation cells were harvested as described above, washed in PBS and fixed in 70% ethanol (Avantor, Poland) at 4°C for 30 minutes. Next, cells were pelleted by centrifugation, resuspended in 1 mL of PBS containing RNase A (10 mg/mL) and incubated at room temperature for 30 minutes. After that, cells were centrifuged and the pellet was suspended in 500  $\mu$ L propidium iodide staining solution (50  $\mu$ g/mL) for 1 hour at room temperature in the dark. Subsequently, cell samples were analyzed with the use of the BD FACSCalibur<sup>™</sup> flow cytometer (Becton Dickinson, USA). For each experiment 10,000 cells were examined. The fluorescence of propidium iodide was excited using an argon laser (488 nm) and emission of red fluorescence was detected in the FL3 channel (>650 nm). Data were collected and analyzed using CellQuest Pro software (v.5.2.1) (Becton-Dickinson). The percentages of cells in the sub  $G_0$ ,  $G_0/G_1$ , S,  $G_2/M$  cycle phases were evaluated using the ModFit<sup>™</sup> software (Verity Software, Inc., USA). After flow cytometric analysis, Modfit software was used to calculate the proliferation index (PI) ( $(S+G_2M/G_0G_1) \times 100$ ) by dividing the percentages of proliferating cells (cells in S and  $G_2/M$  phases) by non-proliferating cells (cells in  $G_0/G_1$  phase).

### Statistical analysis

The data were analyzed using the Statistica 10 software (StatSoft Inc., USA). The distributions of the data were assessed by the Shapiro-Wilk test. Due to nonparametric distributions Kruskal-Wallis test with Dunn's post-hoc test were applied. The data were calculated for three separate experiments and shown as mean  $\pm$  standard deviation (SD). Statistical significance was taken at  $p < 0.05$ .

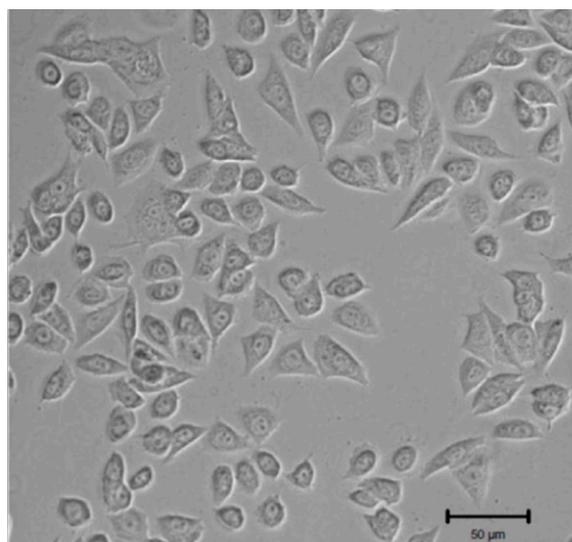
## Results

### Ghsr gene expression analysis

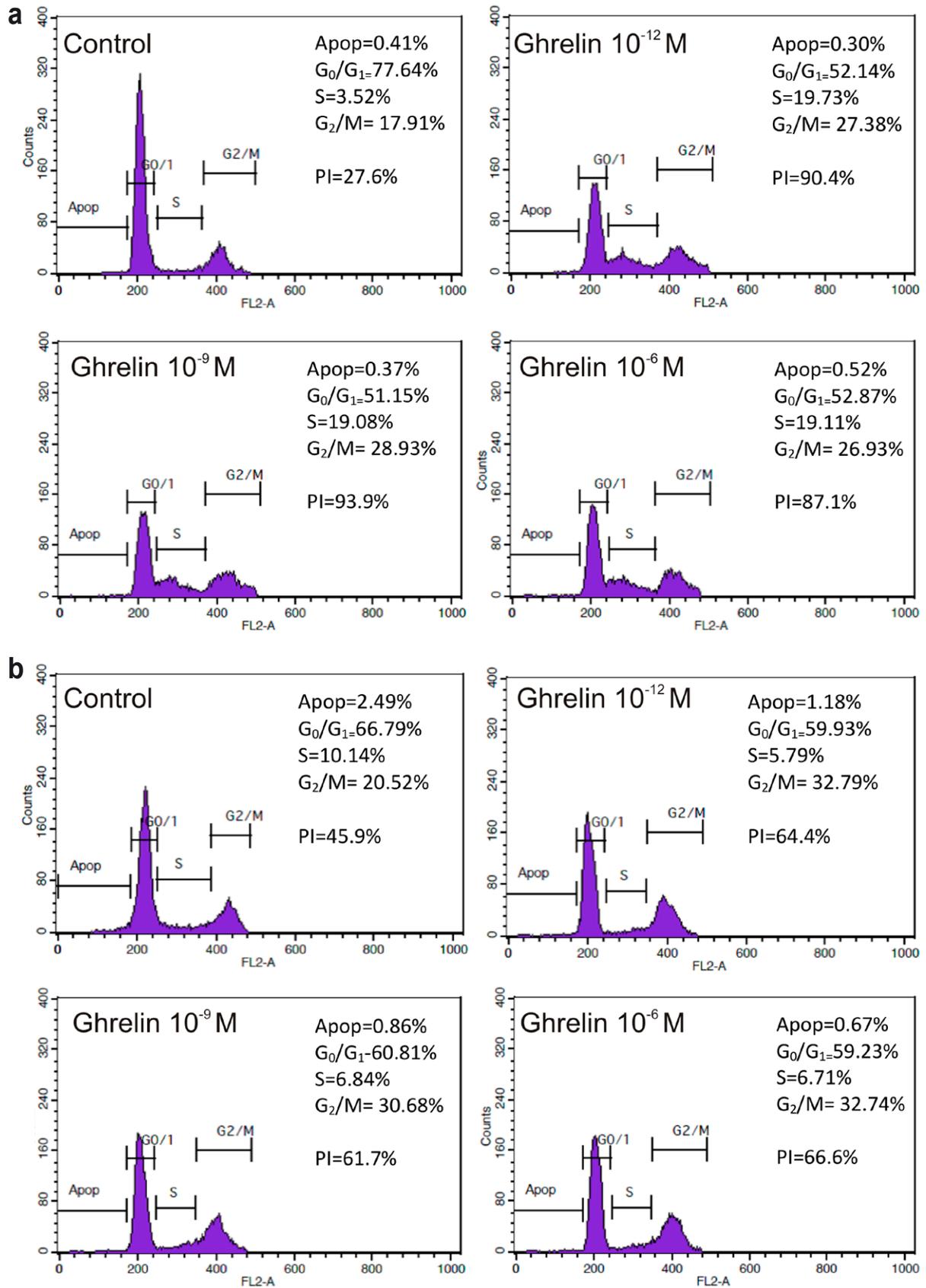
Presence of *Ghsr* gene was confirmed after reverse transcription real time polymerase chain reaction with the use of TaqMan<sup>®</sup> hydrolysis probes by gel electrophoresis (**Figure 1a**) and Sanger sequencing (**Figure 1c**). The size of the electrophoretic separated DNA band as well as alignment (**Figure 1b**) of the sequencing reaction result scored entirely with this deposited in NCBI database and confirmed the identity with *Ghsr* GeneBank N<sup>o</sup> NM\_032075.3

### Ghrelin and GH3 cells morphology

GH3 cells were loosely adherent with floating clusters and exhibited differences in their morphology was culture time-dependent. After the attachment to the Petri dish, the cells were characterized by a spherical shape which has changed to more polygonal with increasing cell confluence (**Figure 2**). Ghrelin did not affect GH3 cells morphology regardless of ligand concentration and incubation time.



**Figure 2.** GH3 pituitary tumor cell line morphology. Representing example of non-stimulated cells

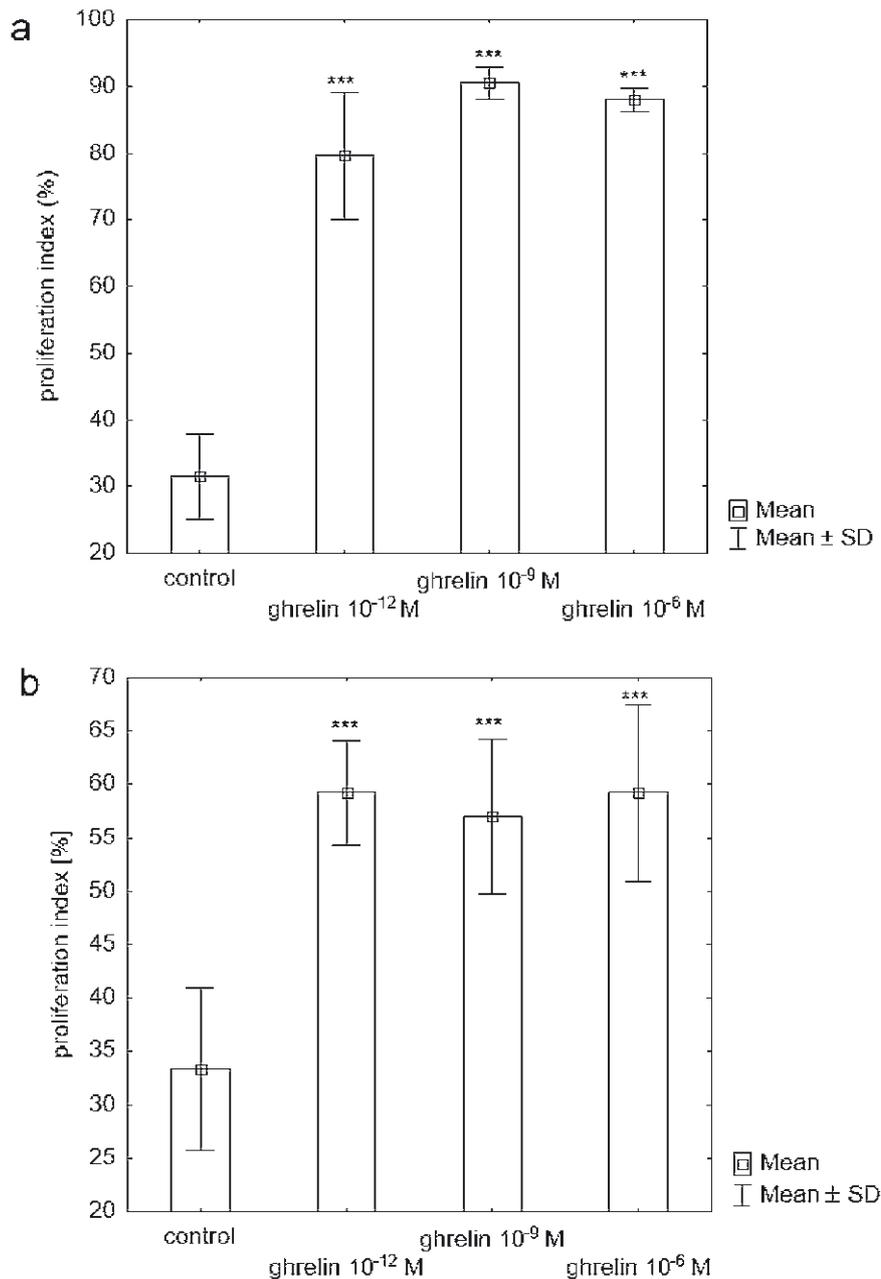


**Figure 3.** Representative example of cell cycle redistribution of GH3 cells after ghrelin stimulation. GH3 cells were incubated with ghrelin in different doses ( $10^{-12}$ ,  $10^{-9}$  or  $10^{-6}$  M) for 24 or 48 h. The cell cycle was analyzed using FACSCalibur™ flow cytometer. The percentages of cells in the sub G<sub>0</sub>, G<sub>0</sub>/G<sub>1</sub>, S, G<sub>2</sub>/M cycle phases were evaluated using the Modfit software. PI – proliferation index ( $S+G_2/M/G_0/G_1 \times 100$ ), Apop – apoptotic cells percentage

### Ghrelin and GH3 cell cycle

In the control group no changes in the cell cycle of GH3 cells were observed during 48 hours observation period ( $p = \text{NS}$ , **Figure 3**). The proliferation index (PI) ranged from 26% to 44% ( $29.3 \pm 3.5\%$ ) after 24 h and 25% to 44% ( $31.2 \pm 6.4\%$ ) after 48 h. Compared to control group, an increase of PI was observed 24 h after stimulation with different ghrelin concentrations:  $29.3 \pm 3.5$  vs  $79.6 \pm 9.5\%$  ( $p < 0.0001$ ) at a concentration  $10^{-12}$  M,  $29.3 \pm 3.5$  vs  $90.5 \pm 2.3\%$  ( $p < 0.0001$ ) at  $10^{-9}$  M and  $29.3 \pm 3.5$  vs  $88.0 \pm 1.8\%$  ( $p < 0.001$ ) at  $10^{-6}$  M. Within the used range

of ghrelin concentrations, we did not observe dose-dependent changes. An increase of PI was also observed 48 h after ghrelin stimulation and was as follows:  $31.2 \pm 6.1$  vs  $59.2 \pm 4.9\%$  ( $p < 0.0001$ ) at a concentration  $10^{-12}$  M,  $31.2 \pm 6.1$  vs  $57.0 \pm 7.3\%$  ( $p < 0.0001$ ) at  $10^{-9}$  M and  $31.2 \pm 6.1$  vs  $59.2 \pm 8.27\%$  ( $p < 0.001$ ) at  $10^{-6}$  M, however, as well as in case of 24 h observation, there were not visible dose-dependent changes (**Figure 3**). Observed increase in the proliferation index was due to both a decrease in  $G_0/G_1$  cell count and the increase in the  $S+G_2/M$  cells' population (**Figure 4**).



**Figure 4.** Dose-dependent effect of ghrelin stimulation in GH3 cells. Proliferation index (a) 24 hours and (b) 48 hours after stimulation. Results are shown as means  $\pm$  standard deviation. Significance is referred to the control (\*\* $P < 0.001$ )

## Discussion

Several studies have demonstrated that ghrelin and its receptors are expressed in pituitary adenomas [8–10]. In this study, we aimed to establish ghrelin influence on somatotrophic cells morphology and proliferation. Ghrelin did not affect GH3 cells morphology regardless of ligand concentration and incubation time. However, ghrelin significantly increased GH3 cells proliferation index, as a result of decreased cells count population in G<sub>0</sub>/G<sub>1</sub> phase and increased S+G<sub>2</sub>/M. In human cells, these effects may be mediated through the GHSR1a, corresponding to this shown to be expressed in GH3 pituitary somatotroph tumor cell line (Ghsr).

Published data regarding ghrelin impact on cells proliferation revealed either stimulation or inhibition. Our results are in line with previously published data concerning GH3 cell line [5, 11] as well as data concerning different cell types of: adrenocortical tumor [12], hepatoma [4], prostate tumors [11], neural [13], pre-adipocytes [14], osteoblasts [12] or cardiomyocytes [15]. Nanzer *et al.* in a study applying <sup>3</sup>H-thymidine incorporation assay demonstrated that ghrelin showed stimulating impact on rat somatotroph pituitary tumor cells proliferation. It was suggested that extracellular signal-regulated kinase (ERK) was involved in this mechanism. Desoctanoyl ghrelin showed a similar effect as octanoylated form [5]. Baldanzi *et al.* suggest that ghrelin and des-acyl ghrelin inhibit cell death through ERK-1/2 an PI3-kinase/AKR. Baldanzi shown that ghrelin stimulates tyrosine phosphorylation and activates ERK-1/2 and Akt [15]. Activation of ERK-1/2 by ghrelin was observed previously by Murata *et al.* [4]. The authors postulated that, independent of its acylation, ghrelin gen products may act as a survival factor [15]. Tian *et al.* demonstrated that nitric oxide (NO) blocked ghrelin-activated GH3 cells proliferation. The mechanism of NO action was mediated by inhibition of extracellular signal-regulated kinase 1/2 [16].

It is postulated that ghrelin impact on GH3 cells proliferation involves Ghsr receptor [5]. The GHSR is encoded by a single-copy gen located on chromosome 3 in humans, whose alternative splicing can generate two mRNA splice variants, *GHSR1a* and *GHSR1b*. The functional activity of GHSR1b remains to be fully elucidated. GHSR1a is the receptor responsible for intracellular acylated-ghrelin transduction signal pathway [17]. However, some authors postulate that ghrelin impact on cells proliferation involves other systems of signal transduction [9, 18–20]. Volante *et al.* described the presence of ghrelin binding sites in cellular membrane of human thyroid neoplastic cells, however the GHSR1a and GHSR1b presence was not observed [19]. Nanzer

*et al.* suggested that ghrelin stimulate cell proliferation directly *via* the MAPK pathway involving the GHSR1a [5].

Anti-proliferative ghrelin effect was described in thyroid [19], breast [2] or pituitary [21] neoplastic cell lines. Either acylated or non-acylated ghrelin was studied [2, 22]. This indicates that non-acylated ghrelin, previously considered inactive due to lack of GH secretion stimulation, manifested its biological activity [12, 15, 21].

We observed that ghrelin stimulated GH3 cell proliferation in a way that was not directly dose-dependent. In fact, after 24h incubation, the maximal stimulatory effect of ghrelin was observed at the 10<sup>-9</sup> M but not at the highest concentration of 10<sup>-6</sup> M. After 48h incubation, the reaction was not also dose-dependent. Maccarinelli *et al.* studied the effect of ghrelin (10<sup>-11</sup> – 10<sup>-8</sup> M) on proliferation and differentiation of osteoblastic cells and observed maximal stimulatory effect at 10<sup>-10</sup> M but not at higher concentrations. Those authors speculate that ghrelin dose increase leads to another receptor subtype representing inhibitory proliferative activity recognition [23]. On the other hand, Tian *et al.* found that ghrelin induced the GH3 cells proliferation in a dose-dependent manner after 48h incubation. They used bromodeoxyuridine (BrdU) assay to determine the proliferation of GH3 cell. Their results revealed that after 48h incubation, ghrelin increased the incorporation of BrdU into GH3 cells in a dose-dependent manner (with significant responses to concentration ranging from 10<sup>-9</sup> M to 10<sup>-6</sup> M) [26].

The proliferation index was assessed to evaluate cell population ratio in each phase of cell cycle. The observed increase of the proliferation index was due to both: decrease of G<sub>0</sub>/G<sub>1</sub> cell count and increase of the S+G<sub>2</sub>/M cells population. In the BrdU method, an analog of the DNA precursor thymidine is incorporated into newly synthesized DNA by the cells entering and progressing through the S (DNA synthesis) phase of the cell cycle. The difference between the techniques may be responsible for the small discrepancy of the results. However, both techniques confirmed stimulating effect of ghrelin on GH3 cells proliferation.

In conclusion, we used GH3 cells as a model of GH-releasing adenoma *in vitro*. We demonstrated that GH3 cell line express *Ghsr* receptor. We showed that ghrelin significantly stimulated GH3 cells proliferation and may play a role in pituitary tumorigenesis *via* an autocrine/paracrine pathway.

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### Conflict of interest statement

The authors declare no conflict of interest.

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