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The Effect of Melatonin on Proliferation of Primary Porcine Cells Expressing Mutated Huntingtin

Vliv melatoninu na proliferaci primárních prasečích buněk exprimujících mutovaný huntingtin

Abstract

According to the recent studies, melatonin might play an important role in Huntington's disease (HD) and act as a novel therapeutic approach in the treatment of the disease. HD, the inherited neurodegenerative disorder, is accompanied by gradual melatonin reduction as it progresses. Melatonin in normal cells (non-tumor) has the anti-apoptotic ability due to its antioxidant property and its ability to prevent the activation of p53. Furthermore, melatonin increases the expression of BDNF (brain derived neurotrophic factor) and other neuroprotective factors. The aim of this study was to evaluate the nontoxic dose of melatonin for primary skin fibroblasts isolated from minipigs transgenic for the N-terminal part of human mutated huntingtin (TgHD), and the effect of melatonin treatment to these cells exposed to genotoxic stress. Cells were cultured in medium supplemented with different doses of melatonin. Using time lapse microscopy, we estimated the effect of decreasing melatonin concentrations by analyzing the proliferation curves. We show that higher doses of melatonin are toxic for primary porcine fibroblasts. Interestingly, TgHD cells were more sensitive to these doses of melatonin treatment than wild type cells. We evaluated the effective dose of melatonin and demonstrated its rescue proliferative effect on porcine primary cells exposed to genotoxic stress.

Souhrn

Současné studie naznačují možnou důležitou úlohu melatoninu v Huntingtonově nemoci (HN) a jeho možné terapeutické využití při léčbě této nemoci. HN je dědičné neurodegenerativní onemocnění, které doprovází snižování hladiny melatoninu s postupem onemocnění. U normálních (nenádorových) buněk působí melatonin antiapoptoticky díky svým antioxidačním vlastnostem a schopnosti zabránit aktivaci proteinu p53. Dále melatonin zvyšuje expresi BDNF (brain derived neurotrophic factor) a dalších neuroprotektivních faktorů. Cílem této studie bylo stanovit netoxickou dávku melatoninu pro primární kožní fibroblasty izolované z transgenních miniprasat pro N-koncovou část lidského mutovaného huntingtinu (TgHD) a popsat efekt tohoto ošetření na tyto buňky vystavené genotoxickému stresu. Buňky byly kultivovány v médiu obohaceném různými dávkami melatoninu. Analýzou proliferačních křivek získaných mikroskopováním živých buněk v pravidelných časových intervalech jsme stanovili efekt různých koncentrací melatoninu. Ukázali jsme, že vyšší dávky melatoninu jsou pro primární prasečí buňky toxické. Je zajímavé, že TgHD buňky byly oproti kontrolním buňkám více citlivé k tímto dávkám melatoninu. Stanovili jsme efektivní dávku melatoninu a současně jsme ukázali její efekt na proliferaci u buněk vystavených genotoxickému stresu.

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Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.
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Key words

Huntington's disease – melatonin – time lapse microscopy – minipig model – proliferation curves – skin fibroblasts

Klíčová slova

Huntingtonova choroba – melatonin – mikroskopie buněk v čase – miniprasečí model – proliferační křivky – kožní fibroblasty

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Aims of the study

Huntington's disease (HD) represents an inherited neurodegenerative disorder lacking an effective treatment. The disease is caused by a gene-coding mutation for the huntingtin protein (mtHtt). HD is not only the disorder affecting the central nervous system, but it also affects the peripheral tissues, because mtHtt is widely expressed throughout the body [1]. There are many other symptoms observed in HD, in addition to neurodegeneration, like osteoporosis, testicular degeneration, and loss of muscle tissue [2].

A prominent feature of the disease is also disturbed sleep [3] caused by progressively declined melatonin levels with advancing disease [4] and significantly reduced levels of melatonin in later stages of HD [5]. The mechanism causing the melatonin secretion disruption remains unknown.

Neurodegeneration in HD is attributed to neural cell death in striatum and cortex of the brain. One of the possible therapeutic strategies targeted to neurodegeneration could represent the reduction of apoptosis [6]. Apoptosis is caused by increased levels of pro-apoptotic protein p53. Increased levels of total p53 and also phosphorylated p53 at serine 15 are observed in brains and peripheral tissues of HD patients as well as in transgenic mouse models of HD [7,8]. Increased levels of p53 phosphorylated at serine 15 [9] are the result of DNA-damage accumulation in HD [10,11].

Melatonin has many physiological functions [12]. It has the ability to induce apoptosis in tumour cells while it prevents it in non-tumour cells. It is an excellent antioxidant that acts as a free radical scavenger and increases the expression and activity of antioxidant enzymes [13]. Melatonin increases the levels of Sirt1 deacetylase in primary neurons. This positively affects the expression of BDNF (Brain Derived Neurotrophic Factor) and other neuroprotective factors and inhibits neurodegeneration in animal models of Alzheimer's and Huntington's disease [14]. There is evidence that melatonin mediates neuroprotection, delays the onset of the disease, and mortality in transgenic mouse models of HD [15]. These findings suggest that melatonin might act as a novel therapeutic approach in the treatment of HD. We generated a unique large animal model for HD, the minipig transgenic for the N-terminal part of human mutated huntingtin (TgHD) [16].

The aim of the study was to follow the melatonin effect on proliferation curves of porcine primary skin fibroblasts derived from TgHD minipigs and their wild type (WT) controls. The first objective was to find the effective nontoxic concentration of melatonin treatment. Furthermore, we evaluated the effect of melatonin in TgHD and WT fibroblasts exposed to genotoxic stress.

Methods

Cell culture

Primary fibroblasts were isolated from skin grafts of miniature pigs transgenic for the N-terminal part of human mutated huntingtin (TgHD) and their age-matched wild type (WT) controls with similar genetic background at the age of 36 months (pre-manifestation phase of HD). Cells were cultured in Minimal Essential Medium α (MEM α) without nucleotides (Gibco, #22561) supplemented with 10% fetal bovine serum (Sigma-Aldrich, #F7524) and 0.1% Gentamicin (Sigma-Aldrich, #G1397) under normal culture conditions (5% CO_2 at 37°C). Cells were cryopreserved in the first passage in MEM α with 10% DMSO (Sigma-Aldrich, #D8418), 25% FBS and stored in liquid nitrogen until use. After thawing the cells in 37°C water bath, cells were cultured as described above under normal culture conditions. All experiments were performed in the third cell passage.

Melatonin treatment

Melatonin (N-acetyl-5-methoxytryptamine) was purchased from Sigma-Aldrich (#M5250) and dissolved in DMSO to obtain a stock solution at the concentration of 0,1M. Additional dilutions were done in culture medium MEM α to achieve the concentrations 10^{-4}M, 10^{-8}M, 10^{-12}M, 10^{-14}M and 10^{-16}M.

Neocarzinostatin treatment

Neocarzinostatin (Sigma-Aldrich #N9162) was added to the medium (1 : 50,000) and immediately added to the cells.

Time-lapse microscopy

Fibroblasts were seeded in the third passage on 12-well plate TPP (5×10^4 cells per well) or

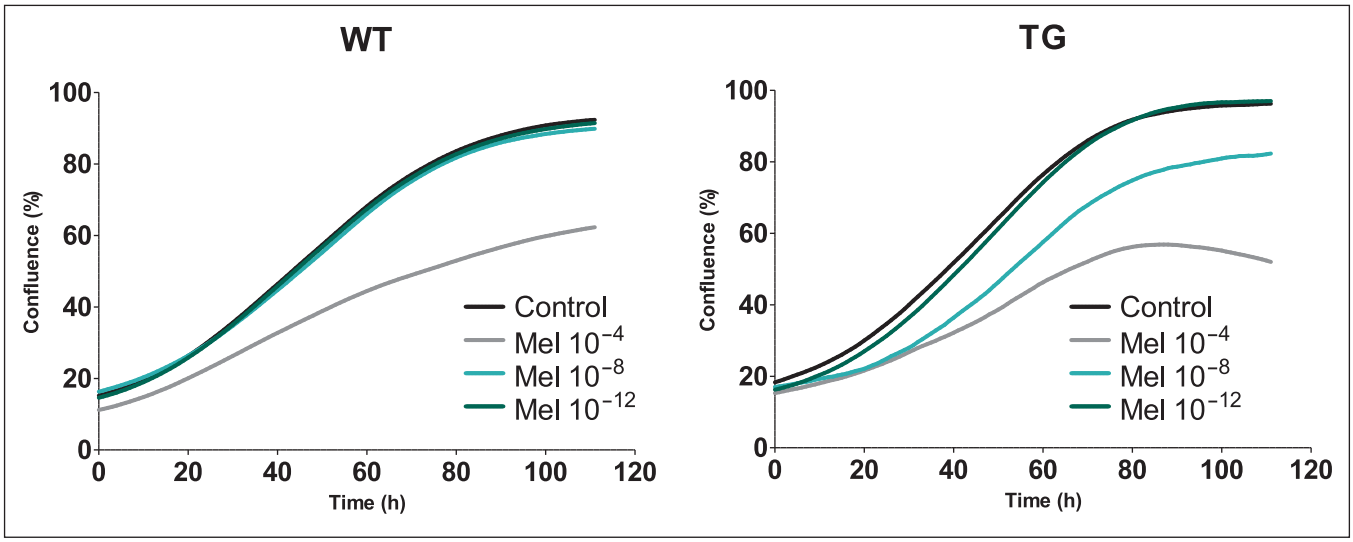


Fig. 1. Time lapse microscopy measurement of the wild type (WT) and TgHD (TG) cell growth on treatment with different doses of melatonin.

Growth curves of control cells in black (control), melatonin treatment (mel) 10^{-4}M in grey, 10^{-8}M in light green and 10^{-12}M in dark green. These data are the mean of three replicates.

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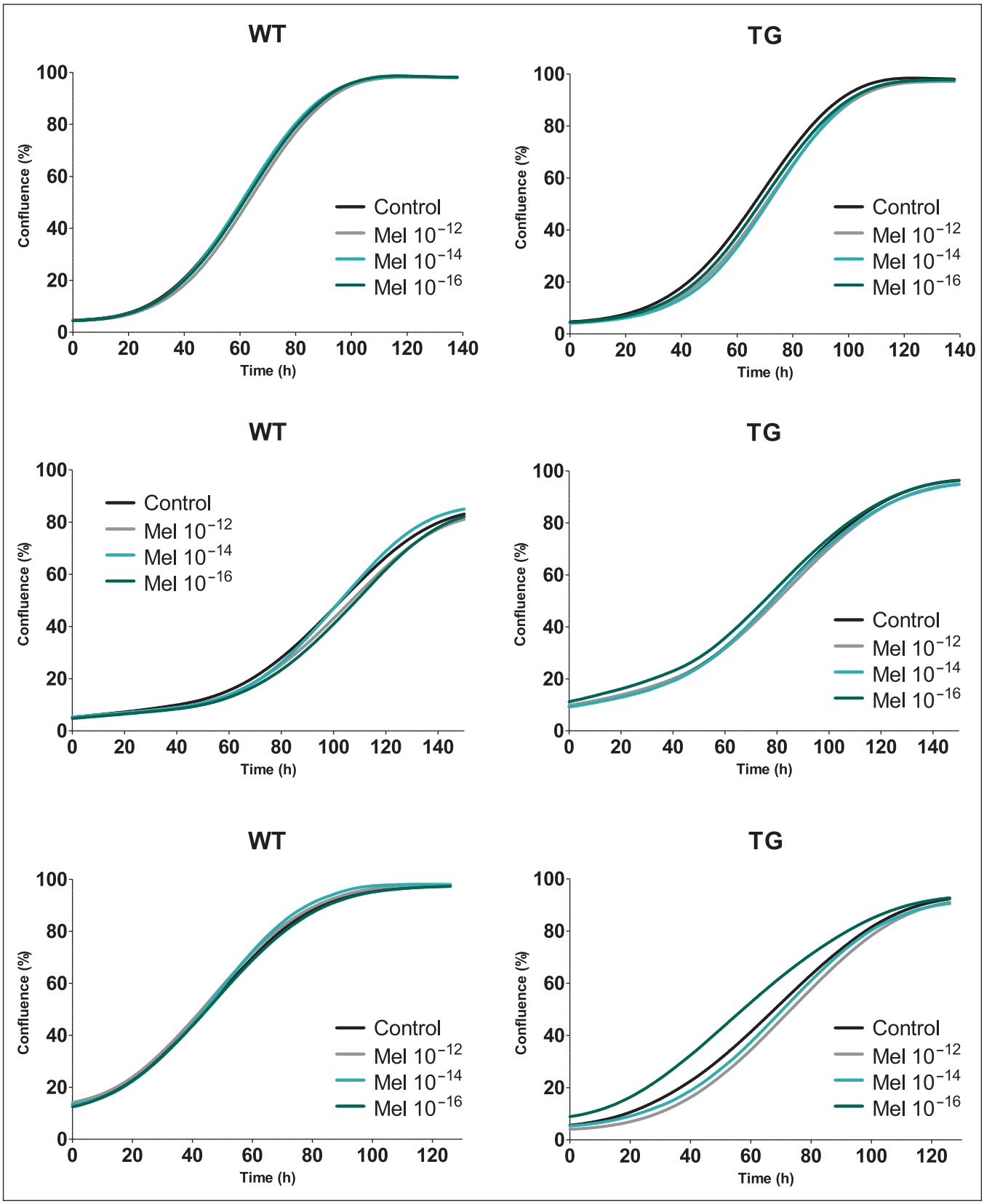


Fig. 2. Time lapse microscopy measurement of the wild type (WT) and TgHD (TG) cell growth on treatment with different doses of melatonin.

Growth curves of control cells in black (control), melatonin treatment (mel) 10^{-12}M in grey, 10^{-14}M in light green and 10^{-16}M in dark green. These data are the mean of three replicates.

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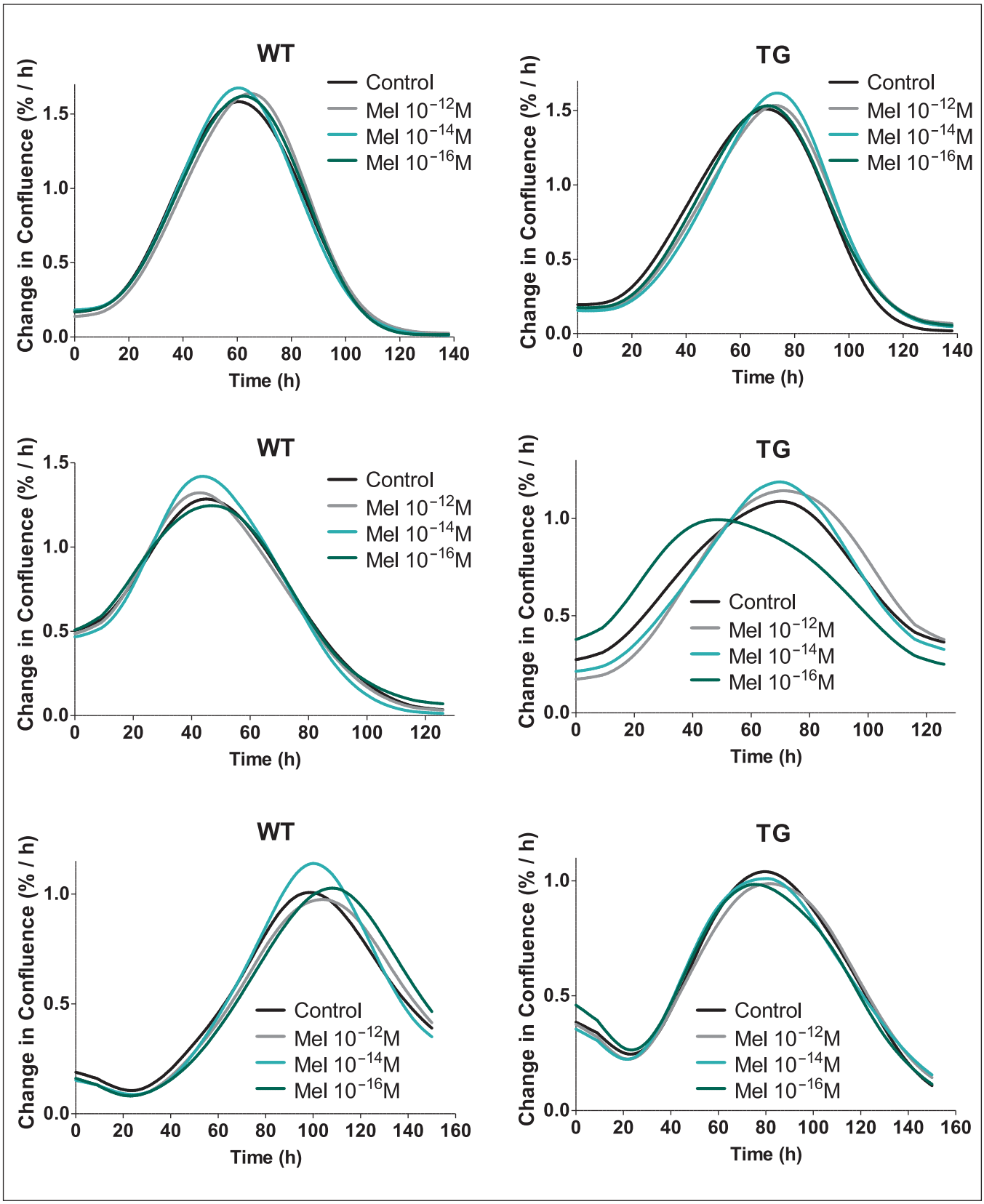


Fig. 3. Change in confluence derived from data of growth curves of the wild type (WT) and TgHD (TG) cell on treatment with different doses of melatonin.

Control cells in black (control), melatonin treatment (mel) 10^{-12}M in grey, 10^{-14}M in light green and 10^{-16}M in dark green. These data are the mean of three replicates.

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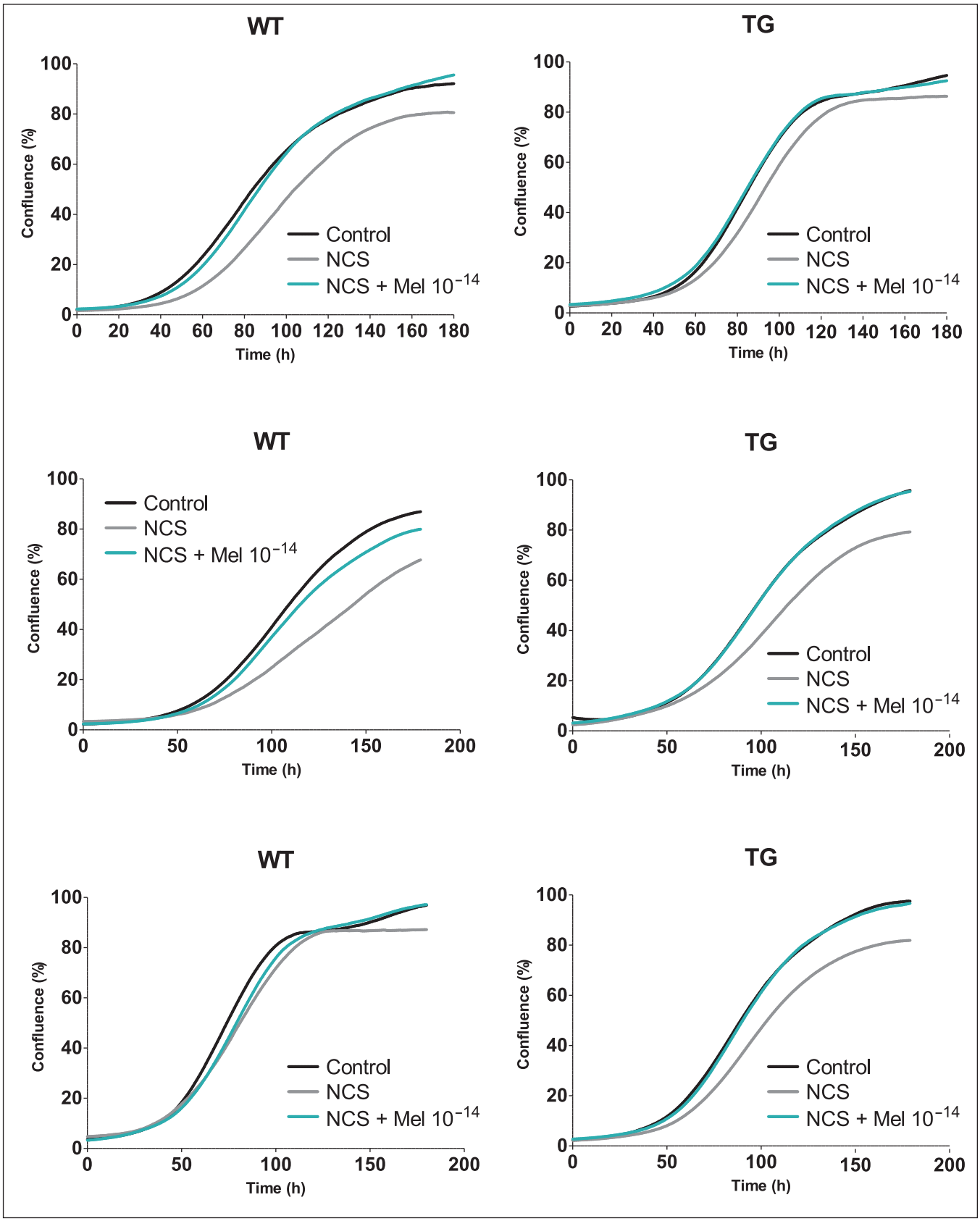


Fig. 4. Time lapse microscopy measurement of the wild type (WT) and TgHD (TG) cell growth on treatment with neocarzinostatin (NCS, 1 : 50,000) in grey, neocarzinostatin (NCS, 1 : 50,000) and melatonin (mel) 10^{-14}M in light green and control cells (control) in black.

These data are the mean of two replicates.

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24-well plate TPP (2×10^4 cells per well) and cultured six hours under normal culture conditions, while the cells were attached to the surface of the well. After incubation period, media were exchanged with corresponding media containing different doses of melatonin, melatonin and neocarzinostatin (NCS), and normal culture medium MEM α to the control cells.

Cells were imaged each hour by time-lapse microscopy using IncuCyte (Essen Bioscience, Welwyn Garden City, UK) with phase contrast and scan pattern 4 × 4 under normal culture conditions (5% CO_2 at 37°C) for at least five days.

Data analysis

The cell confluence was calculated using the IncuCyte software (2009B, Rev2) as a mean of three replicates or two replicates (NCS treatment). To determine the maximum speed of the cell proliferation the rate of change curves, derived from cell confluence curves, was performed.

Results

In order to evaluate the optimal concentration of melatonin treatment for primary cells isolated from TgHD and age-matched WT miniature pigs we treated cells with different doses of melatonin ranging from 10^{-4}M to 10^{-12}M and scanned them by time-lapse microscopy to obtain cell growth curves (Fig. 1).

Application of melatonin at the concentration 10^{-4}M resulted in marked inhibition of TgHD and WT cell proliferation. Interestingly, addition of lower concentration of melatonin (10^{-8}M) inhibited TgHD, but did not inhibit the WT cell growth, suggesting a higher sensitivity of TgHD cells to stress. No changes of cell proliferation compared to the control were detected after incubation with the concentration of melatonin 10^{-12}M.

After showing that the dose 10^{-12}M is not toxic for primary porcine cells, we decided to measure the effect of lower doses of melatonin ranging from 10^{-12}M to 10^{-16}M (Fig. 2). Addition of melatonin at different concentrations (10^{-12}M, 10^{-14}M and 10^{-16}M) to the TgHD and WT cells did not inhibit cell proliferation compared to untreated control cells.

In order to choose one concentration of melatonin treatment we further examined the change in confluence derived from proliferation curves of melatonin treatment 10^{-12}M, 10^{-14}M and 10^{-16}M demonstrated the highest peak of the speed of cell growth at 10^{-14}M melatonin treatment in five from six investigated animals (Fig. 3).

Next, we investigated the effect of 10^{-14}M melatonin on cells exposed to genotoxic stress induced by neocarzinostatin, radiomimetic drug causing DNA double strand breaks (Fig. 4).

Neocarzinostatin caused decreasing cell growth due to DNA damage. In contrast, treating the stressed cells with melatonin 10^{-14}M resulted in the enhancement of the cell proliferation. This indicates that the treatment with melatonin 10^{-14}M of porcine cells exposed to genotoxic stress causes a rescue effect and may play a role in preventing the DNA damage.

Conclusion

In this study, we tested the effect of melatonin on porcine TgHD and WT fibroblasts exposed to genotoxic stress. The effect of melatonin on stimulation or suppression of cell division depends on its concentration and cell type examined [17], and it has not yet been published in porcine skin fibroblasts. We demonstrated that the dose of melatonin 10^{-4}M is toxic for primary porcine fibroblasts. Moreover, we found that TgHD cells are more sensitive to 10^{-8}M dose of melatonin treatment than WT cells. This shows a higher responsiveness of TgHD cells to stress. We showed that the concentrations of melatonin treatment 10^{-12}M 10^{-14}M and 10^{-16}M are not harmful for primary cells isolated from miniature pigs transgenic of the N-terminal part of human mutated huntingtin. Nevertheless, based on change in confluence derived from proliferation curves, we chose the concentration of 10^{-14}M as an optimal concentration of melatonin treatment for porcine fibroblasts. Previous findings showed that melatonin prevents DNA-damage by promoting DNA repair [18]. Using the concentration of 10^{-14}M, we demonstrated that melatonin treatment of porcine cells exposed to genotoxic stress caused rescue effect in WT as well as in TgHD cells, which suggests a role of melatonin in preventing DNA-damage in Huntington's disease.

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