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Molekularne mechanizmy aktywności nanocząstek srebra na poziomie komórkowym

Molecular mechanisms of silver nanoparticles activity on cellular level

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STRESZCZENIE

WSTĘP

Nanocząstki obecne w środowisku, zarówno pochodzenia naturalnego i antropogeniczne, mają wpływ na ludzkie zdrowie. Powszechne zastosowanie nanomateriałów w wielu gałęziach przemysłu skutkuje wzrastającą akumulacją nanocząstek w środowisku abiotycznym, oraz w tkankach organizmów żywych. Negatywnymi skutkami biologicznej aktywności nanocząstek wskazywanymi najczęściej są alergie, nowotwory, choroby układu naczyniowo-sercowego, zaburzenia w rozwoju embrionalnym, zaburzenia wzrostu, toksyczność względem układu immunologicznego, zaburzenia krzepnięcia krwi, toksyczność w barierze krew-mózg, choroby neurodegeneracyjne, oraz zapalne dróg oddechowych i choroby z nimi związane[1].

Reaktywne formy tlenu i reaktywne formy azotu są produkowane w komórkach w warunkach fizjologicznych, jako cząsteczki gwarantujące homeostazę wewnątrzkomórkową. Odgrywają one kluczową rolę w procesie transdukcji sygnałów wewnątrzkomórkowych.

Istotnym czynnikiem powodującym toksyczny efekt nanocząsteczek jest stres oksydacyjny, definiowany jako zaburzenie w równowadze między produkcją reaktywnych form tlenu i obroną antyoksydacyjną. Stres oksydacyjny związany jest z zaburzeniami komórkowego metabolizmu takimi jak zaburzenia w funkcjonowaniu łańcucha oddechowego i/lub glikolizy, wobec czego, stężenie glukozy jest jednym z kluczowych czynników warunkujących homeostazę redoks.

Zwiększenie ilości glukozy w środowisku zewnątrzkomórkowym powoduje zmianę metabolizmu energetycznego komórek z fosforylacji oksydacyjnej na glikolizę, co może

zwiększyć produkcję reaktywnych form tlenu w komórce. To zjawisko jest tłumaczone zwiększeniem wewnątrzkomórkowego stężenia jonów wapnia, co skutkuje rozpadem mitochondriów i prowadzi do apoptozy. Dane literaturowe uzyskane w badaniach na liniach komórkowych są zbieżne z doświadczeniami z udziałem pacjentów cierpiących na cukrzycę typu drugiego, u których wykryto znaczącą utratę komórek β tłumaczoną zahamowaniem czynności glukozo-6-fosfatazy[2]. Metabolizm energetyczny większości komórek prawidłowych, w warunkach fizjologicznych, jest uzależniony od fosforylacji oksydacyjnej natomiast metabolizm komórek nowotworowych bazuje głównie na glikolizie[3]. To zjawisko, nazywane efektem Warburga, jest formą adaptacji komórek nowotworowych rozwijających się w warunkach hipoksji. Ograniczenie funkcji mitochondriów sprzyja jednocześnie ograniczeniu apoptozy, co jest jednym z objawów transformacji nowotworowej komórek.

Stres azotowy jest silnie powiązany ze stresem oksydacyjnym. Pierwotny rodnik azotowy – tlenek azotu jest syntezowany z udziałem argininy, tlenu i NADPH przez syntazy tlenku azotu. Mimo krótkiego czasu półtrwania, wynoszącego kilka milisekund, cząsteczki tlenku azotu dyfundują bez przeszkód przez błony biologiczne, dzięki czemu są jednymi z kluczowych, autokrynnych i parakrynnych cząsteczek sygnałowych u kręgowców. Uniwersalność tlenku azotu jako cząsteczki sygnałowej, warunkuje jej udział w procesach na poziomie organizmalnym, takich jak regulacja napięcia mięśni gładkich naczyń krwionośnych i odpowiedź immunologiczna, oraz na poziomie narządów i komórek. W przypadku wątroby niewielkie stężenie tlenku azotu ma na celu utrzymanie homeostazy oraz zapobieganie stanom patologicznym. Jest ono warunkowane ekspresją konstytutywnej syntazy tlenku azotu. Natomiast indukowalna syntaza tlenku azotu ulega ekspresji w zaburzeniach funkcjonowania komórek wątroby[4]. Tlenek azotu jest cząsteczką o ograniczonej reaktywności, która w reakcji z anionorodnikiem ponadtlenkowym tworzy nadtlenoazotyn. Nadtlenoazotyn jest z kolei efektywnym utleniaczem i czynnikiem nitrującym, którego obecność w komórce skutkuje obniżeniem stężenia wewnątrzkomórkowych antyoksydantów oraz uszkodzeniami białek, lipidów i kwasów nukleinowych.

Stres oksydacyjny jest zjawiskiem o dużym znaczeniu dla utrzymania homeostazy wewnątrzkomórkowej. Jest to związane nie tylko z potencjalnymi uszkodzeniami oksydacyjnymi cząsteczek budujących komórkę, ale również z rolą regulacyjną jaką pełnią wolne rodniki i inne reaktywne formy tlenu. Kluczowe szlaki przekaźnictwa wewnątrzkomórkowego mogą być regulowane przez status redoks komórek, jak również może dochodzić do bezpośredniej aktywacji czynników transkrypcyjnych pod wpływem reaktywnych form tlenu. Aktywacja

czynników transkrypcyjnych będzie w końcowym efekcie prowadziła do zmian w profilu ekspresyjnym genów i powstawania nowych białek. Dobrze udokumentowanym przykładem aktywacji czynnika transkrypcyjnego bezpośrednio przez status redoks jest aktywacja czynnika Nrf2, który ma zdolność do aktywacji genów, które w sekwencji promotorowej zawierają element ARE, a także genów dla białek odpowiedzialnych za detoksykację, np. transferaz glutationowych. Wiele białek z nadrodziny ABC jest pod kontrolą czynników transkrypcyjnych zależnych od statusu redoks. Zaburzenia ekspresji transporterów ABC mogą mieć znaczenie dla prawidłowego funkcjonowania komórki. W przypadku wystąpienia procesów chorobowych, takich jak nowotworzenie, mogą mieć znaczenie dla powodzenia stosowanego leczenia.

CELE PRACY

1. Ocena endogennej produkcji reaktywnych form tlenu zależnej od aktywności łańcucha oddechowego, jako czynnika modulującego toksyczność nanocząstek srebra.
2. Rozstrzygnięcie roli stresu azotowego w toksyczności nanocząstek srebra.
3. Ocena roli generowanych pod wpływem nanocząstek wolnych rodników tlenowych w procesie modulacji profilu mRNA dla wybranych białek ABC w dojrzałych dopaminergicznych neuronach oraz neuronach niezróżnicowanych.

METODY

Do realizacji celów rozprawy wykorzystano dwa komórkowe modele badawcze. Korzystano z linii HepG2 (linia wyprowadzona z raka wątrobowokomórkowego), oraz LUHMES (linia komórkowa wywodząca się ze śródmózgowia 8-tygodniowego płodu). Komórki linii HepG2 hodowano w medium DMEM w dwóch wariantach stężenia glukozy. Niższe stężenie (5,5 mmol/dm^3) odpowiadało warunkom fizjologicznym, natomiast stężenie 25 mmol/dm^3 jest zbieżne ze stężeniem wykrywanym podczas ostrego epizodu hiperglikemii. Linia LUHMES stanowi model neuronów dla komórek dzielących się, a po różnicowaniu służy jako model dojrzałych dopaminergicznych neuronów. Różnicowanie komórek LUHMES w dojrzałe dopaminergiczne neurony przeprowadzono zgodnie z procedurą opublikowaną w pracy Stępkowski T i wsp., 2016[5]. Obie linie komórkowe zostały zakupione z kolekcji ATCC (American Type Culture Collection). Hodowle komórkowe prowadzono zgodnie z zaleceniami. Jako czynniki inicjujący odpowiedź biologiczną modeli badawczych wykorzystano nanocząstki srebra o średnicy nominalnej 20 nm. Ocenę przeżywalności przeprowadzono w oparciu test akumulacji czerwieni obojętnej w lizosomach komórek żywych. Pomiary wewnątrzkomórkowej produkcji anionorodnika ponadtlenkowego w mitochondriach i cytoplazmie, nadtlenku wodoru oraz

tlenku azotu wykonano przy użyciu cytometrii przepływowej w oparciu o fluorescencję specyficznych sond. Pomiary produkcji mitochondrialnego nadtlenku wodoru wykonano z użyciem białka reporterowego HyPer-mito i cytometrii przepływowej.

Izolacja kwasów nukleinowych została przeprowadzona za pomocą urządzenia MagNA Pure LC 2.0 (HepG2) oraz z użyciem odczynnika TRI (LUHMES). Reakcje odwrotnej transkrypcji przeprowadzono z użyciem SuperScript™ III First-Strand Synthesis SuperMiX. Ekspresja genów na poziomie mRNA związanych ze stresem oksydacyjnym była badana z użyciem komercyjnie dostępnego zestawu starterów (HOSL-1 – The human oxidative stress library). Ekspresja genów na poziomie mRNA dla białek z rodziny ABC została oceniona z użyciem komercyjnie dostępnego zestawu starterów (Roche). Stężenie nitrotyrozyny zostało oznaczone za pomocą komercyjnie dostępnego zestawu immunochemicznego ELISA (Merck Milipore), natomiast poziom nitracji tyrozyny w układzie bezkomórkowym został zbadany przez pomiar intensywności fluorescencji nitrotyrozyny. Aktywność enzymów antyoksydacyjnych została wyznaczona poprzez pomiar szybkości zmian w stężeniu odpowiednich substratów klasycznymi metodami absorpcjometrycznymi.

WYNIKI

Badania przedstawione w ramach rozprawy zawarte są w trzech powiązanych tematycznie pracach. W pracach *Glucose availability determines silver nanoparticles toxicity in HepG2* oraz *Silver nanoparticles can attenuate nitrative stress* stwierdzono, że zmiana stężenia dostępnej glukozy w medium hodowlanym skutkowała ustaleniem nowej równowagi redoks. Stabilny status redoks, komórki uzyskiwały po 72 godzinach od momentu zmiany stężenia czynnika modulującego aktywność mitochondriów (glukozy). Ocenie poddano aktywność enzymów związanych z obroną antyoksydacyjną w komórkach hodowanych w medium suplementowanym różnymi stężeniami glukozy. Proces adaptacji komórek do obniżonego stężenia glukozy w środowisku spowodował wzrost znaczenia fosforylacji oksydacyjnej w energetycznym metabolizmie komórek. Obserwowany wzrost aktywności katalazy, S-transferazy glutationowej, dysmutazy ponadtlenkowej i reduktazy glutationowej świadczy o procesie adaptacji komórek do nowego stanu równowagi redoks. Zbadany został profil ekspresji genów związanych z obroną antyoksydacyjną w komórkach hodowanych na niższym stężeniu glukozy w stosunku do poziomu kontrolnego. Proces adaptacyjny komórek linii HepG2 został potwierdzony przez analizę transkryptomu odpowiedzialnego głównie za odpowiedź komórki na stres oksydacyjny. Zwiększonej ekspresji uległy geny NOS2, GSTM5, ALB, MBL2, SCARA3 i CAT. Jednocześnie zaobserwowano obniżenie ekspresji genów związanych

z metabolizmem glutationu (GSS, GSTZ1, GSTA4, GPX), co sugeruje, że detoksykacja reaktywnych form tlenu produkowanych w następstwie obniżenia stężenia glukozy zachodzi na drodze szybkich, enzymatycznych reakcji, a nie z udziałem drobnocząsteczkowych antyoksydantów. Ponadto, stopniowe i chroniczne obniżenie stężenia glutationu aktywuje szlak przekaźnictwa NFκB, co kieruje metabolizm komórki na drogę indukcji mechanizmów antyoksydacyjnych.

Obserwacje te pozwoliły zaproponować mechanizm, który decydował o różnicach w przeżywalności komórek HepG2 pod działaniem nanocząstek srebra w zależności od zawartości glukozy w medium hodowlanym.

Stężenie glukozy w medium hodowlanym jest czynnikiem modulującym toksyczność nanocząstek srebra. Mechanizm tego zjawiska polega na stymulacji zdolności obrony antyoksydacyjnej komórek hodowanych w medium o obniżonym stężeniu glukozy przez intensyfikację fosforylacji oksydacyjnej związaną ze zwiększoną produkcją reaktywnych form tlenu. Śmierć 10 % komórek w hodowli prowadzonej z użyciem stężenia glukozy standardowo używanego w hodowli komórek HepG2 (25 mmol/dm^3) wywołały nanocząstki srebra w stężeniu 1,4 raza niższym niż w przypadku hodowli utrzymywanej na medium o obniżonym poziomie glukozy (5,5 mmol/dm^3) przez 24 godziny i 7,6 raza niższym niż w przypadku komórek hodowanych w medium o obniżonym poziomie glukozy przez miesiąc i dłużej.

Oceniono produkcję nadtlenku wodoru w mitochondriach oraz produkcję reaktywnych form tlenu i azotu w komórkach HepG2 hodowanych w medium suplementowanym różnymi stężeniami glukozy w obecności nanocząstek srebra.

Zaobserwowano, że nanocząstki srebra wywołują stres oksydacyjny w komórkach HepG2, objawiający się, zwiększeniem stężenia H_2O_2 w mitochondriach o 18 % w stosunku do komórek kontrolnych. Hodowla komórek w medium o obniżonym stężeniu glukozy umożliwiła adaptację komórek do warunków podwyższonego stresu oksydacyjnego dzięki zwiększonej aktywności mitochondrialnego łańcucha transportu elektronów, wobec czego nie zaobserwowano podwyższonego poziomu H_2O_2 po dodaniu do komórek nanocząstek srebra.

Obniżenie stężenia glukozy w medium hodowlanym spowodowało spadek wykrywanych ilości rodnika ponadtlenkowego na poziomie komórkowym i mitochondrialnym, oraz obniżenie poziomu nadtlenków wewnątrzkomórkowych. Nanocząstki srebra spowodowały wzrost poziomu mitochondrialnego anionorodnika ponadtlenkowego oraz nadtlenków w komórkach hodowanych na medium o wyższym stężeniu glukozy. Poziom badanych

reaktywnych formy tlenu nie uległ zmianie pod wpływem nanocząstek srebra w komórkach hodowanych z użyciem medium o niższym stężeniu glukozy.

Aby ocenić czy nanocząstki srebra modulują nitrację tyrozyny w układzie bezkomórkowym wykonano doświadczenia *in vitro*. Wykazano, że nanocząstki srebra obniżają poziom nitracji tyrozyny, najprawdopodobniej przez przyspieszanie rozkładu nadtlenoazotynu.

Zweryfikowano hipotezę o roli reaktywnych form azotu i produktów stresu azotowego poprzez ocenę stężenia nitrotyrozyny w komórkach HepG2 hodowanych w mediach o różnym stężeniu glukozy, w obecności nanocząstek srebra.

Stwierdzono, że endogenny poziom nitrotyrozyny w komórkach HepG2 był poza zasięgiem detekcji zestawu ELISA użytego w doświadczeniach. Nitracja białek wywołana egzogennym nadtlenoazotynem osiągnęła wyższy poziom w komórkach hodowanych w medium o obniżonym stężeniu glukozy. Nitrotyrozyna była szybciej usuwana w komórkach hodowanych w medium o wyższym stężeniu glukozy, co jest związane z większą aktywnością proteasomu. Podobny efekt zaobserwowano w komórkach hodowanych w medium o niższym stężeniu glukozy poddanych działaniu nanocząstek srebra, które na drodze szlaku przeźnictwa sygnałów NRF2 również powodują wzrost aktywności proteasomu.

W wyżej wymienionych pracach wykazaliśmy, że toksyczność nanocząstek srebra jest silnie zależna od statusu redoks komórek, który jest konsekwencją aktywności łańcucha mitochondrialnego. Pozwoliło, to na postawienie tezy, że komórki których metabolizm silnie zależy od glikolizy są bardziej podatne na uszkodzenia wywołane działaniem nanocząstek srebra. Przykładem takich komórek są komórki neuronalne. Celem dalszych doświadczeń była ocena w jaki sposób stres oksydacyjny wywołany przez nanocząstki srebra może wpływać na profil mRNA genów kodujących białka z rodziny ABC. Białka z nadrodziny ABC są bardzo istotne dla prawidłowego funkcjonowania neuronów, warunkując równowagę lipidową w komórkach neuronalnych. Zmiana poziomu tych białek jest wiązana z procesem różnicowania oraz prawidłowym funkcjonowaniem przekaźnictwa wewnątrzkomórkowego.

W pracy *Exposure of human neurons to silver nanoparticles induces similar pattern of ABC transporters gene expression as differentiation: Study on proliferating and post-mitotic LUHMES cells* największe zmiany w transkryptomie badanych genów zaobserwowano dla genów z rodziny ABCA, między innymi dla genu ABCA1. Zapostulowano mechanizm, w którym internalizacja fragmentów błony bogatych w cholesterol towarzysząca endocytozie nanocząstek srebra, zwiększa pulę cholesterolu w komórce dostępnego dla reaktywnych form tlenu. Oksysterole będące efektem utleniania cholesterolu są czynnikiem aktywującym czynnik

transkrypcyjny LXR, który warunkuje homeostazę cholesterolową w mózgu oraz wpływa na zwiększenie ekspresji białka ABCA1.

Stwierdzono, że komórki intensywnie proliferujące poddane działaniu nanocząstek srebra wykazują profil ekspresji mRNA dla białek ABC analogiczny do tego, który związany jest z procesem różnicowania się w dojrzałe neurony dopaminergiczne. Świadczyć to może o tym, że stres wywołany przez nanocząstki w komórkach proliferujących może powodować zaburzenia różnicowania w płodowych komórkach neuronalnych.

WNIOSKI

1. Dostępność glukozy moduluje toksyczność nanocząstek srebra. Jest to związane z uaktywnieniem mechanizmów obrony antyoksydacyjnej przez wzmożoną aktywność łańcucha mitochondrialnego i co za tym idzie zwiększoną produkcję reaktywnych form tlenu.
2. Typy komórek silnie zależne od glikolizy są bardziej podatne na toksyczny efekt nanomateriałów indukujących stres oksydacyjny.
3. Nanocząstki srebra nie wywołują stresu azotowego, ale wpływają na potranslacyjne modyfikacje białek pośrednio, przez wpływ na ekspresję białek związanych z obroną przed stresem nitracyjnym.
4. Prekursorowe komórki neuronalne są bardziej podatne na zmiany w ekspresji genów dla białek ABC wywołane przez nanocząstki srebra niż zróżnicowane neurony dopaminergiczne.
5. Proces różnicowania neuronów powoduje podobny profil zmian w ekspresji genów dla białek ABC jak aktywność biologiczna nanocząstek srebra.
6. Nanocząstki srebra aktywują czynnik transkrypcyjny LXR, co skutkuję zwiększoną ekspresją genu ABCA1 i możliwymi zaburzeniami w homeostacie cholesterolu.

SUMMARY

INTRODUCTION

Nanoparticles present in the environment, both natural and anthropogenic, have a significant impact on human health. Wide range of nanomaterial application in various branches of industry results in their increasing accumulation in abiotic environment and in tissues of living organisms. Most often mentioned negative effects of nanoparticles biological activity are: inflammation of the respiratory tract and respiratory diseases, allergies, cancer, cardiovascular diseases, developmental toxicity, immune toxicity, toxicity to organs, thrombosis, toxicity in the blood-brain barrier and neurodegenerative diseases[1].

Both, reactive oxygen species and reactive nitrogen species are produced in cells as an important element of cellular physiology. They play a crucial regulatory role due to their involvement in intracellular signal transduction.

Oxidative stress is an important factor in producing undesirable effects by nanoparticles. Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses or, more recently, as disruption of redox signaling and control. Occurrence of the oxidative stress is often associated with disturbances in metabolic processes, such as deregulation of mitochondrial respiratory chain and/or glycolysis.

Especially, concentration of glucose has a significant effect on cellular metabolism, as increased glucose level results in switching of cells metabolism from oxidative phosphorylation (OXPHOS) to glycolysis in various cell types, that in turn increases ROS production. A proposed mechanism for this phenomenon involves an increase in intracellular calcium concentration resulting in mitochondrial fission through the function of dynamin-like protein 1, that leads to apoptosis. Results of *in vitro* studies on the link between increased glucose concentration and inhibition of cell proliferation in the model cell lines correlate with *in vivo* results. In patients with type 2 diabetes a massive loss of beta-cells is observed, which is associated with oxidative stress induced by inhibition of glucose-6-phosphatase[2]. In physiological conditions majority of normal cells rely on OXPHOS, whereas cancer cells metabolism is based mostly on glycolysis (so-called Warburg effect)[3]. The Warburg effect may be an adaptation to the limited oxygen supply, as an early development of cancer cells usually takes place in hypoxic environment of the growing tumor that has limited blood supply until its own vasculature is developed. Limiting the function of mitochondria can also promote the reduction of apoptotic process probability, which is one of the symptoms of carcinogenesis.

The primordial nitrogen radical – nitric oxide is synthesized from arginine, oxygen and NADPH by nitric oxide synthases. Despite the short half-life of a few milliseconds, nitric oxide molecules diffuse freely through the biological membranes, which makes them one of the crucial, both autocrine and paracrine, signaling molecules in vertebrates. The universality of nitric oxide as an intermittent signal molecule determines its participation in many processes at the organismal level, such as the regulation of the smooth muscle tone in the blood vessels and contribution in the process of immune response. In the liver, a small concentration of nitric oxide is key factor in maintaining homeostasis and prevention of pathologies. The structural concentration of nitric oxide is maintained in the course of activity of constitutive nitric oxide synthase. In contrast, the inducible nitric oxide synthase is expressed in liver cells of disturbed functions[4]. Nitrative stress is strongly connected to oxidative stress. Primal nitrogen radical – nitric oxide, is a particle of mild reactivity. However, its reaction with superoxide anion produces, peroxynitrite a molecule effective as oxidant and nitrating agent. Peroxynitrite, due to its aforementioned properties depletes cellular antioxidants and damages proteins, lipids and nucleic acids.

Oxidative stress is a phenomenon of great importance for maintaining intracellular homeostasis. This is not only connected with potential oxidative damage of cell-building molecules, but also to the regulatory role played by free radicals and other reactive oxygen species. The key pathways of intracellular signal transmission can be regulated by the redox status of the cell, as well as through direct activation of transcription factors by reactive oxygen species. The activation of transcription factors will ultimately lead to changes in the gene expression profile. A well-documented example of activating a transcription factor directly through the redox status is activation of Nrf2 which has the ability to activate genes that contain the ARE element in the promoter sequence as well as genes coding for proteins responsible for the detoxification, such as GST. Many of the proteins from the ABC superfamily are under control of transcription factors dependent on the redox status. Disorders in the expression of ABC transporters can be important for the proper functioning of cells and may be important in the course of treatment of cancers.

In course of my research I focused on silver nanoparticles-induced oxidative and nitrative stress, with special attention devoted to mitochondria. The aim of this study was to extend the knowledge in the aspect of mechanism of action of nanoparticles on the cellular level.

AIMS

1. Evaluation of endogenous production of reactive oxygen species dependent on the activity of the respiratory chain, as a factor modulating toxicity of silver nanoparticles.
2. Resolving the role of nitrogen stress in the toxicity of silver nanoparticles.
3. Evaluation of the role of oxygen free radicals generated under the influence of nanoparticles in the process of modulation of mRNA profile for selected ABC proteins in mature dopaminergic neurons and undifferentiated neurons.

METHODS

Two cell lines were used as research models to achieve the goals of this dissertation: HepG2 cell line – derived from hepatocellular carcinoma and LUHMES cell line – derived from the midbrain of 8-week fetus. HepG2 cells were grown in DMEM medium in two variants of glucose concentration. The lower concentration (5.5 mmol / dm^3) corresponded to the physiological conditions, whereas the concentration of 25 mmol / dm^3 is convergent with the concentration in acute diabetic shock. The LUHMES cell line is a model of dividing and mature dopaminergic neurons. Differentiation of LUHMES cells into mature dopaminergic neurons was performed according to work of Stepkowski et al [5]. Both cell lines were purchased from ATCC (American Type Culture Collection). Cell cultures were carried out in accordance with recommendations. Silver nanoparticles with a nominal diameter of 20 nm were used as agents initiating biological response. Survival assessment was based on the neutral red accumulation in lysosomes of living cells. Measurements of intracellular superoxide anion generation in the mitochondria and cytoplasm, hydrogen peroxide and nitric oxide levels were performed using flow cytometry readings employing specific probes. Measurements of the production of mitochondrial hydrogen peroxide were conducted using the HyPer-mito reporter protein with flow cytometry techniques.

Isolation of nucleic acids was carried out using the MagNA Pure LC 2.0 device (HepG2) and TRI reagent (LUHMES). Reverse transcription reactions were perforrmed using SuperScript III First-Strand Synthesis SuperMIX. Gene expression associated with oxidative stress on mRNA level was tested using commercially available set of primers (HOSL-1 – The human oxidative stress library). Gene expression of ABC family proteins on mRNA level was evaluated using a commercially available set of primers (Roche). The concentration of nitrotyrosine was determined using commercially available ELISA kit (Merck Milipore), while the level of tyrosine nitration in the cell-free system was examined by measuring the fluorescence intensity of

nitotyrosine. The activity of antioxidant enzymes was determined by measuring the rate of changes in level of appropriate substrates with classical absorption spectroscopy methods.

RESULTS

The research presented in this dissertation is contained in three thematically related papers. In *Glucose availability determines silver nanoparticles toxicity in HepG2* and *Silver nanoparticles can attenuate nitrative stress*, it was shown that the change in the concentration of available glucose in the culture medium resulted in the establishment of new redox balance. Stable redox status of the cells was attained 72 hours after the change in concentration of glucose as mitochondria activity modulating factor. The activity of enzymes related to antioxidant defense in cells grown in media supplemented with different glucose concentrations was evaluated. The adaptation process of cells to reduced concentration of glucose in the environment caused an increase in the importance of oxidative phosphorylation in cells metabolism. The observable increase in catalase, glutathione S-transferase, superoxide dismutase and glutathione reductase activities indicates the process of cells adaptation to new redox equilibrium. In addition, the expression profile of genes related to antioxidant defense was examined in cells grown at a lower glucose level. The adaptation process of the HepG2 cells was confirmed by the analysis of the transcriptome responsible mainly for the cell response to oxidative stress. The NOS2, GSTM5, ALB, MBL2, SCARA3 and CAT genes have been upregulated. At the same time, the decrease in the expression of genes related to glutathione metabolism (GSS, GSTZ1, GSTA4, GPX) suggests that the detoxification of reactive oxygen species produced as a result of lowering glucose concentration is carried out through rapid enzymatic reactions and not by small antioxidant molecules. In addition, gradual and chronic reduction of glutathione levels activates NFκB signal transmission pathway, which induces antioxidative response.

These observations allowed us to propose a mechanism that was decisive in the differences in survival of HepG2 cells cultured in varying glucose concentrations after treatment with silver nanoparticles.

The concentration of glucose in the culture medium is a factor modulating the toxicity of silver nanoparticles. Reducing the concentration of glucose intensified oxidative phosphorylation accompanied by increase in reactive oxygen species production, which in turn, resulted in elevating intracellular antioxidative defense mechanisms. The death of 10 % of cells in high glucose culture was caused by silver nanoparticles in concentration 1.4 times lower than

in case of culture sustained in low glucose for 24 hours and 7.6 times lower for culture sustained in low glucose for a month or more.

HepG2 cells were studied in context of production of hydrogen peroxide in the mitochondria and the production of reactive oxygen and nitrogen species in cytoplasm

It has been observed that silver nanoparticles caused oxidative stress in HepG2 cells, manifested by an increased concentration of H_2O_2 in the mitochondria by 18% in comparison to control cells. Culturing cells in a medium with reduced glucose concentration enabled the adaptation of cells to the conditions of increased oxidative stress due to increased activity of mitochondrial electron transport chain, so that no increase of H_2O_2 level was observed after treatment with silver nanoparticles.

Reduction of glucose concentration in the culture medium resulted in decrease in detected superoxide radicals at the cellular and mitochondrial level as well as decrease in intracellular peroxides. Silver nanoparticles caused an increase in the level of the mitochondrial superoxide anion and peroxides in cells grown in a medium with a higher concentration of glucose. The level of tested reactive oxygen species did not change after treatment with silver nanoparticles in cells cultured in a medium of lower glucose concentration.

To assess whether silver nanoparticles modulate tyrosine nitration in a cell-free system, *in-vitro* experiments were performed. Silver nanoparticles have been shown to lower the level of tyrosine nitration, most likely by accelerating the decomposition of peroxynitrite.

The hypothesis about the role of reactive nitrogen species and nitrogen stress product was verified by the assessment of nitrotyrosine concentration in HepG2 cells cultured in media with various concentration of glucose in the presence of silver nanoaprticles.

It was found that the endogenous level of nitrotyrosine in HepG2 cells was beyond the detection range of the used ELISA kit. Protein nitration caused by exogenous peroxynitrite increased was higher in cell cultured in low glucose medium. Nitrotyrosine was removed faster in cells cultured in a medium with a higher concentration of glucose, which is associated with higher proteasome activity. A similar effect was observed in cells grown in a medium with lower glucose concentration treated with silver nanoparticles, which, through activation of NRF2 signaling pathway also cause an increase in proteasome activity.

In the above mentioned works we have shown that the toxicity of silver nanoparticles is strongly dependent on the cellular redox status, which is a consequence of the activity of the mitochondrial chain. This allowed the thesis that the cells whose metabolism strongly depends on glycolysis are more susceptible to damage caused by the action of silver nanoparticles,

neuronal cells being an example. The purpose of further experiments was to assess how the oxidative stress induced by silver nanoparticles can affect the mRNA profile of ABC proteins. The proteins from the ABC superfamily are very important for the proper functioning of neurons, conditioning the lipid balance in neuronal cells. The change in the level of these proteins is associated with the process of differentiation and the proper functioning of intracellular signal transmission.

In *Exposure of human neurons to silver nanoparticles induces similar pattern of ABC transporters gene expression as differentiation: Study on proliferating and post-mitotic LUHMES cells* biggest changes in the transcriptome of studied genes were observed for genes coding proteins from the ABCA family. A mechanism was proposed where the internalization of cholesterol-rich membrane fragments caused by endocytosis of silver nanoparticles increased the pool of cholesterol available for reactive oxygen species generated by silver nanoparticles. Oxysterols resulting from cholesterol oxidation can activate LXR, which determines the cholesterol homeostasis in the brain and increases the expression of the ABCA1 protein.

It has been found that intensely proliferating cells treated with silver nanoaprticles have similar pattern of expression profile changes as cells during differentiated into mature dopaminergic neurons. This may indicate that stress induced by nanoparticles in proliferating cells may cause disturbances of differentiation in fetal neuronal cells.

CONCLUSIONS

1. The availability of glucose modulates the toxicity of silver nanoparticles. This is related to the activation of antioxidant defense mechanisms through the increased activity of the mitochondrial chain and, consequently, increased production of reactive oxygen species.
2. Cell types strongly dependent on glycolysis are more susceptible to the toxic effect of nanomaterials inducing oxidative stress.
3. Silver nanoaprticles do not induce nitrogen stress, but affect post-translational protein modifications indirectly, by affecting the expression of proteins associated with protection against nitrative stress.
4. Neuronal precursor cells are more susceptible to changes in gene expression for ABC proteins induced by silver nanoparticles than differentiated dopaminergic neurons.
5. The process of neuronal differentiation results in a similar profile of changes in gene expression of ABC proteins as the biological activity of silver nanoparticles.

6. Silver nanoparticles activate LXR transcription factor which results in increased expression of the ABCA1 gene and possible disturbances in the cholesterol homeostasis.

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RESEARCH

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Glucose availability determines silver nanoparticles toxicity in HepG2

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Abstract

Background: The increasing body of evidence suggest that nanomaterials toxicity is associated with generation of oxidative stress. In this paper we investigated the role of respiration in silver nanoparticles (AgNPs) generated oxidative stress and toxicity. Since cancer cells rely on glucose as the main source of energy supply, glucose availability might be an important determinant of NPs toxicity.

Methods: AgNPs of 20 nm nominal diameter were used as a model NPs. HepG2 cells were cultured in the media with high (25 mM) or low (5.5 mM) glucose content and treated with 20 nm AgNPs. AgNPs-induced toxicity was tested by neutral red assay. Generation of H_2O_2 in mitochondria was evaluated by use of mitochondria specific protein indicator HyPer-Mito. Expression of a 77 oxidative stress related genes was assessed by qPCR. The activity of antioxidant enzymes was estimated colorimetrically by dedicated methods in cell homogenates.

Results: AgNPs-induced dose-dependent generation of H_2O_2 and toxicity was observed. Toxicity of AgNPs towards cells maintained in the low glucose medium was significantly lower than the toxicity towards cells growing in the high glucose concentration. Scarceness of glucose supply resulted in upregulation of the endogenous antioxidant defence mechanisms that in turn alleviated AgNPs dependent ROS generation and toxicity.

Conclusion: Glucose availability can modify toxicity of AgNPs via elevation of antioxidant defence triggered by oxidative stress resulted from enhanced oxidative phosphorylation in mitochondria and associated generation of ROS. Presented results strengthen the idea of strong linkage between NPs toxicity and intracellular respiration and possibly other mitochondria dependent processes.

Keywords: Nanosilver, Oxidative stress, Antioxidant enzymes activity, Warburg effect

Background

Nanoparticles (NPs) present in the environment, both of natural origin or anthropogenic, may have a significant impact on human health. Presence of the NPs in the body causes pathophysiological changes that might contribute to the development of cancer [1], cardiovascular diseases [2], respiratory tract inflammation [3], neurodegenerative diseases [4, 5], and many other pathologies [6].

Silver NPs (AgNPs) are among the one of the widest use in everyday life products, despite their reported adverse effects against various cell lines and organisms. The accumulating body of evidence suggests that harmful action of AgNPs is associated with the induction of oxidative stress [7, 8].

Oxidative stress is the most often described as an imbalance in cellular production and consumption of reactive oxygen species (ROS). Although ROS play an important role in many physiological processes, the redox imbalance is associated with many pathologies, such as Parkinson's disease [9], Crohn's disease, skin disease mediated by T cells, diabetes, cancer, Leigh syndrome and other mitochondrial diseases. Occurrence of the oxidative stress is often associated with disturbances in metabolic processes, such as deregulation of mitochondrial respiratory chain and/or glycolysis. Especially,

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concentration of glucose has a significant effect on cellular metabolism, as increased glucose level results in switching of cells metabolism from oxidative phosphorylation (OXPHOS) to glycolysis in various cell types [10], that in turn increases ROS production [11]. A proposed mechanism for this phenomenon involves an increase in intracellular calcium concentration resulting in mitochondrial fission through the function of dynamin-like protein 1 [12], that leads to apoptosis [13]. It was reported that just 2-times higher glucose concentration (50 mM) than this used in standard high-glucose DMEM leads to apoptosis of HepG2 cells [14]. Results of in vitro studies on the link between increased glucose concentration and inhibition of cell proliferation in the model cell lines correlate with in vivo results. In patients with type 2 diabetes a massive loss of beta-cells is observed, which is associated with oxidative stress induced by inhibition of glucose-6-phosphatase [15].

In physiological conditions majority of normal cells rely on OXPHOS, whereas cancer cells metabolism is based mostly on glycolysis (so-called Warburg effect) [16]. The Warburg effect may be an adaptation to the limited oxygen supply, as an early development of cancer cells usually takes place in hypoxic environment of the growing tumor that has limited blood supply until its own vasculature is developed, or be due to the shutting down of mitochondria to prevent the apoptosis [17].

Since many reports describe induction of oxidative stress by NPs and there is a number of premises linking the toxicity of NPs to oxidative stress [18, 19], with particular emphasis on ROS generation in the mitochondria, it is of great interest to ascertain, whether the availability of glucose can modify NPs toxicity. Thus the aim of this study was to determine how the glucose availability and associated changes in redox balance will affect the toxicity of NPs.

Results

Silver nanoparticles characteristic

Nanoparticle hydrodynamic diameter and zeta potential was measured by DLS. Hydrodynamic diameter of AgNPs of nominal size 20 nm was approximately 8 times higher when measured in albumin containing buffer. The increase of NP's hydrodynamic diameter in protein containing medium is well recognized phenomenon usually ascribed to formation of protein corona [20]. High negative zeta potential value (−47.6 mV) confers high stability of the AgNPs dispersion and its resistance to aggregation. This was further confirmed by longitudinal analysis. Neither hydrodynamic diameter, zeta potential nor polydispersity index changed markedly over the 2 h that indicate no agglomeration (Table 1). Similar AgNPs behaviour and dispersion stability was previously observed in different cell culture media [21].

Silver nanoparticles toxicity in high- and low-glucose culture medium

Figure 1 shows the survival of HepG2 cells cultured in high- or low-glucose DMEM for 30 days or 24 h, followed by a 24 h-treatment with AgNPs. Comparison of the survival curves reveals a statistically significant protective effect of long term culture in low glucose medium against AgNPs induced toxicity. Transferring cells to low glucose medium for 24 h before the AgNPs treatment resulted in the similar, but yet smaller, not significant effect.

The subsequent analysis of cell survival curves showed an decreasing difference in the effective concentrations of AgNPs necessary to induce toxicity at the particular level, between cells cultured on high- and low-glucose DMEM and cells cultured in low glucose medium for the long and short time (Table 2). The effective concentration of AgNPs, which resulted in 90 % survival of HepG2 cells cultured for 1 month in low glucose medium, was 5.5-times higher than this for cells incubated in low glucose DMEM for 24 h and 7.6-times higher than this for cells maintained in regular, high glucose DMEM. On the contrary, the predicted effective concentration of AgNPs, which resulted in 10 % survival of long term low glucose cells was only 1.4-fold higher than those for short term low glucose or long term high glucose. In preliminary experiments, we checked if the different glucose content in the culture medium affects the growth rate of HepG2 cells. No statistically important differences were found (Student's "t" test, data not shown).

Oxidation of reporter protein with extracellular H_2O_2

In order to quantify the reactivity and sensitivity of the HyPer-mito protein in our system with respect to H_2O_2, we treated the HyPer-mito-transfected cells with exogenous H_2O_2 and measured the fluorescence of the HyPer-mito protein. We found a hyperbolic dependence of the fluorescence on the concentration of H_2O_2, with a saturation effect over 100 μM H_2O_2 (Fig. 2). Thus, the concentration of 25 μM was chosen as a positive control for the further experiments, as the one enabling the upward and downward modification of fluorescence values.

Generation of H_2O_2 in mitochondria of HepG2 cells

Figure 3 presents generation of H_2O_2 in mitochondria of HepG2 cells cultured in different glucose concentrations and treated with AgNPs. H_2O_2 generation in mitochondria of cells cultured on regular, high glucose medium treated with AgNPs was higher than in control cells by 20 %, while the positive control (25 μM extracellular H_2O_2) was greater by 40 % (Fig. 3a). Figure 3b

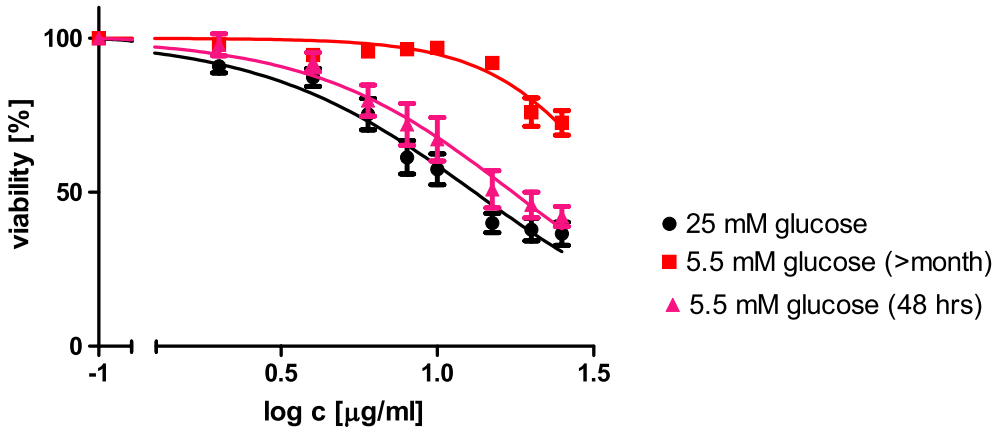

Fig. 1 Toxicity of AgNPs (2.5–50 µg/cm^3) for HepG2 cells cultured in the high- or low-glucose medium. Curves were compared with extra sum of square F test, p = 0.0007, N = 12 (5.5 mM glucose for 30 days, 25 mM glucose) and 9 [5.5 mM glucose (48 h)]. *Graphed points* represent means with SD

Table 1 Hydrodynamic diameter, zeta potential and aggregation over 2 h of silver nanoparticles used in this work of nominal size 20 nm

Time (h)	Hydrodynamic diameter (nm)	Polydispersity index	Zeta potential (mV)
0	160.3 ± 2.1	0.31 ± 0.04	−47.6 ± 0.5
0.5	156.1 ± 5.3	0.31 ± 0.03	
1	160.5 ± 3.4	0.30 ± 0.01	
2	151.8 ± 4.2[a]	0.30 ± 0.03	

Results are presented as a mean ± SD. Means were compared by Student's *t* test (n = 3)

[a] Denotes statistically important difference from the control (time 0 h)

presents corresponding data for cells maintained on low glucose medium for 30 days. No statistically significant difference in H_2O_2 generation by mitochondria was observed between control cells and AgNPs-treated ones. While, the positive control showed only 16 % increase in HyPer-mito protein derived fluorescence, as compared with control cells. In control cells, without AgNPs, sustained on media with lower glucose concentration higher fluorescence intensity can be observed than in those sustained on media with high glucose content.

Expression of oxidative stress related genes

To further examine the mechanism underlying different susceptibility of HepG2 cultured on low- and high-glucose medium to AgNPs, the expression of genes related to the cellular response to oxidative stress was evaluated (Table 3). A marked increase of expression of several genes coding proteins directly involved in oxidative defence was observed. Among those, the most notable change was observed for *ALB* (8.6-fold upregulation), *CAT* (3.5-fold upregulation), *GLRX* (2.3-fold upregulation), *GPX3* (2.2-fold upregulation), *GSTM3* (2.2-fold upregulation), *GSTM5* (72-fold upregulation), *GSTT1* (2.6-fold upregulation), *MBL2* (4.5-fold upregulation), *NCF1* (2.9-fold upregulation), and *SCARA3*

Table 2 The AgNP concentrations necessary to induce cell death at the given survival level

Dead cells %	AgNP concentrations necessary to induce cell death at the given survival level (µg/cm^3)		
	High glucose culture	Low glucose culture (24 h)	Low glucose culture (30 days)
10[b]	1.81 ± 0.41	2.52 ± 0.57	13.84 ± 2.05
25[b]	4.60 ± 0.58	5.82 ± 0.73	22.91 ± 1.93
50[b]	11.70 ± 0.93	13.45 ± 1.15	37.94 ± 7.63
75[a]	29.75 ± 4.46	31.06 ± 4.99	62.83 ± 22.235
90[a]	75,61 ± 19.35	71.77 ± 19.33	104 ± 21.63

Values of each IC were compared by ANOVA accompanied by Tukey's post test

Values represent means ± half of respective confidence intrerval, α = 0.05, n = 3

[a] Denotes statistically important difference between low glucose (30 days) and other cultures

[b] Means that viability of each culture differs from other two

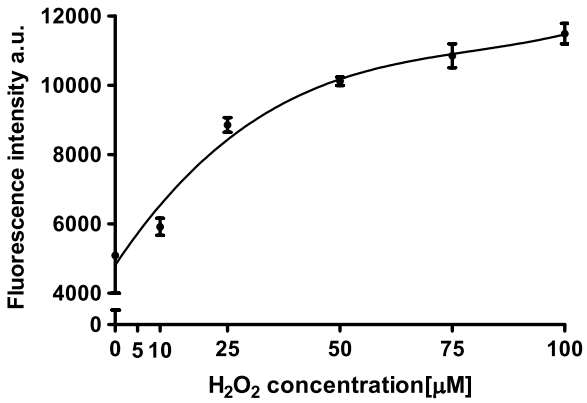

Fig. 2 Influence of extracellular H_2O_2 on fluorescence intensity of reporter protein HyPer-Mito in HepG2 cells cultured on high glucose medium. *Marked points* represent mean ± SD, N = 3

(3.5-fold upregulation). Interestingly, several gene were downregulated. The most significantly down-regulated genes include: *CSDE1, GSS, OXSR1, AOX1, SRXN1, KRT1, GSTZ1, GSTA4, GPX7, SOD1, NCF2, TXNRD2, EPX, PRNP, CYGB, MPV17, PRDX3, NME5, PREX1*. Noteworthy, several of down regulated genes encode proteins involved in the glutathione synthesis and metabolism.

The expression of 33 genes (among 72 studied) was not affected the by long term culture on the low glucose medium. These genes include *ALOX12, ANGPTL7, APOE, ATOX1, BNIP3, DHCR24, DUSP1, EPHX2, FOXM1, GLRX2, GPR156, GPX1, GPX2, GPX4, GSR, GSTP1, LPO, MGST3, MSRA, MT2A, OXR1, PDLIM1, PRDX2, PEDX4, PRDX5, PRDX6, PTGS1, RNF7, SEPP1, SIRT2, SOD3, STK25, TXNRD1*(Additional file 1) .

Activity of key antioxidant defence enzymes

In addition to the transcriptome analysis, enzymatic activity of the proteins playing a crucial role in the cellular antioxidant defence was evaluated (Table 4). Catalase activity was elevated 1.19 times, whereas Zn-Cu superoxide dismutase activity was elevated 1.37 times. Glutatione S-transferase and glutatione reductase activities were elevated 1.69 and 1.4 times, respectively.

Discussion

HepG2 cells are a well established model for study of the effects of glucose on cellular metabolism and response to different stimuli, as these cells can be easily grown in both, low- and high-glucose media [22]. Being a cell line derived from the liver, one of the target organs for nanoparticle mediated toxicity, HepG2 cells are also a well recognized model for nanotoxicity testing, including AgNPs toxicity. Toxicity of AgNPs has been demonstrated across different systems ranging from the model cell lines in vitro to small animals. In the present study, cell viability was evaluated by the Neutral red assay. This assay is less prone to artefacts than the commonly used MTT assay, as the reduction of tetrazolium dyes is strongly dependent on the metabolic state of the cell, in particular on the activity of NAD(P)H-dependent oxidoreductases [15]. Neutral red viability test is based on dye accumulation in lysosomes of viable cells and seems to be less dependent on fluctuations in the redox state of the cells. However, despite the test used, published results repeatedly confirm toxicity of AgNPs [8]. Also the current study confirmed the toxicity of AgNPs to HepG2 cells in vitro.

Analyzing the survival curves for HepG2 cells cultivated in media with different glucose concentration and treated with AgNPs we observed an increase in resistance

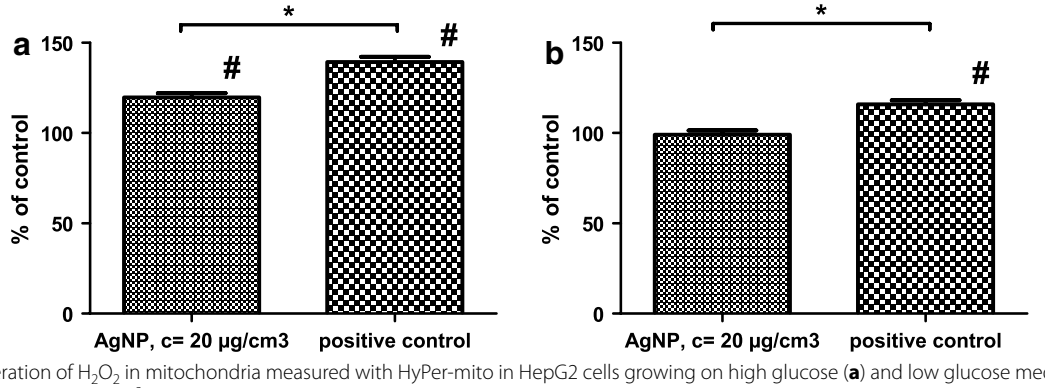

Fig. 3 Generation of H_2O_2 in mitochondria measured with HyPer-mito in HepG2 cells growing on high glucose (**a**) and low glucose medium (**b**) in the presence of 25 μg/cm^3 AgNPs. Extracellular H_2O_2 (25 μM) was used as a positive control. Results were normalized versus control (untreated cells) and statistical importance of differences was calculated with ANOVA accompanied by Tukey`s post hoc test. # indicates statistically important difference from the respective control, $p < 0.05$, N = 3. * denotes statistically important difference between AgNPs treated cell and the respective positive control, $p < 0.05$, N = 3. Values represent means with SD

Table 3 Expression of genes involved in redox regulation in cells

Up-regulation						Down-regulation					
Gene	Expression	95 % CI	Gene	Expression	95 % CI	Gene	Expression	95 % CI	Gene	Expression	95 % CI
GSTM5	72,004	67.62–76.43	GPX3	2.199	1.88–2.48	CSDE1	0.801	0.69–0.92	NCF2	0.626	0.57–0.69
ALB	8.574	8.17–9.24	DUOX1	1.853	1.29–2.68	GSS	0.79	0.619–0.990	TXNRD2	0.564	0.48–0.67
MBL2	4.511	3.92–5.50	EPHX2	1.832	1.66–2.13	OXSR1	0.765	0.63–0.97	EPX	0.354	0.16–0.65
SCARA3	3.547	1.32–5.23	NUDT1	1.803	1.26–2.57	AOX1	0.758	0.64–0.85	PRNP	0.334	0.27–0.42
CAT	3.531	2.47–5.30	PRDX-1	1.713	1.32–2.22	SRXN1	0.734	0.56–0.89	CYGB	0.325	0.29–0.36
NCF1	2.908	1.52–5.91	PNKP	1.295	1.14–1.49	KRT1	0.704	0.53–0.98	MPV17	0.289	0.20–0.42
GSTT1	2.688	2.32–3.25	NUDT2	1.275	1.12–1.51	GSTZ1	0.702	0.53–0.92	PRDX3	0.227	0.09–0.56
GSTM3	2.389	1.94–3.03	TTN	1.254	1.12–1.43	GSTA4	0.686	0.60–0.82	NME5	0.138	0.09–0.19
SGk2	2.335	2.09–2.55	CYBA	1.24	1.17–1.32	GPX7	0.657	0.58–0.77	PREX1	0.108	0.07–0.20
GLRX	2.303	1.22–6.16	GSTM2	1.143	1.03–1.32	SOD1	0.657	0.45–0.92			

Results are presented as fold change of expression in HepG2 cells cultured on low glucose relative to expression in HepG2 cells cultured on high glucose along with 95 % confidence intervals. Only statistically important results were combined in this table ($p < 0.05$)

Table 4 Enzymatic activity of the key players of cellular antioxidative defence

Enzyme	Activity (u/mg protein)	
	25 mM glucose	5.5 mM glucose
Catalase	75.19 ± 6.62	89.81 ± 7[a]
Glutathione S–transferase	0.97 ± 0.16	1.64 ± 0.26[a]
Superoxide dismutase	582 ± 75.2	803 ± 87.5[a]
Glutathione reductase	9.21 ± 1.59	12.94 ± 1.19[a]

Enzyme activities were compared with t test ($n = 4$, $\alpha = 0.05$)

[a] Denotes statistical important difference between HepG2 cells cultured on high glucose medium and low glucose medium for 30 days. Values represent means with standard deviation

to the NPs in cells cultured on low glucose for 30 days, as compared to cells cultured on high glucose medium or on low glucose medium for 24 h (Fig. 1). This effect is especially visible at the high survival levels, e.g. the AgNPs concentrations necessary to reduce the survival of HepG2 cells growing on low glucose medium for 30 days to 90 and 75 % were approximately 7.6 and 5 times higher than those necessary to reduce the survival of the cells growing on high glucose medium to the same survival level, respectively (Table 2). Interestingly, the difference decreases along with the decrease of the survival level, as the estimated AgNPs concentrations necessary to reduce the survival of HepG2 cells growing on low glucose medium for 30 days to 25 and 10 % were only 2.1 and 1.4 times higher than those necessary to reduce the survival of cells growing on high glucose medium to the same survival level, respectively (Table 2). There was no difference between cells growing on low glucose medium for 24 h and those growing on high glucose medium. These results clearly point to the existence the adaptive mechanisms in low-glucose-growing cells that diminish the toxic effect of AgNPs, but also indicate a limited capacity of the adaptive mechanisms, as the difference between the low- and high-glucose growing cells decreased with the decrease of the survival level.

Although the mechanisms of the AgNPs toxicity is still under debate, it is most likely associated with the induction of oxidative stress. Induction of the oxidative stress by AgNPs has been proven in different systems in vitro and in vivo [7, 8, 22]. It is usually associated with excessive generation of free radicals due to the dysfunction of mitochondria [23] and/or activation of ROS generating enzymes, e.g. NADPH reductase [24]. Whereas fluorescent probes commonly used to assess ROS generation are either non-specific or difficult to target to the particular cell compartment, in this work we directly and specifically confirmed generation of H_2O_2 in mitochondria of AgNPs treated HepG2 cells, by the use of mitochondria targeted, H_2O_2 specific HyPer-mito reporter protein. AgNPs treatment of HepG2 cells growing on the standard high glucose medium resulted in the increase of H_2O_2 generation in the mitochondria by 40 %, as compared with control, untreated cells (Fig. 3a). This is consistent with the previous report on AgNP dependent ROS generation in HepG2 cells [21], and the report of generation of H_2O_2 in bronchial epithelial cells (BEAS-2B) [25]. In contrast, AgNPs treatment did not increase generation of H_2O_2 in HepG2 cells grown on low-glucose medium, in concordance with the survival data described above. A plausible, coming to the mind explanation of this phenomenon is an intensification of the antioxidant defence systems that diminish AgNPs-induced ROS toxicity. In parallel with this interpretation, treatment with exogenous H_2O_2 resulted in an only 16 % increase in H_2O_2 detected inside mitochondria of cells grown on low glucose, whereas the same treatment resulted in 40 %

increase of H_2O_2 detectable in mitochondria of the high glucose growing cells.

Indeed, analysis of the enzymatic activity of the key enzymes of the cellular antioxidant defence system revealed statistically significant increase of the activities of all tested enzymes, including catalase, glutathione S-transferase, glutathione reductase and superoxide dismutase (Table 4). While the acute oxidative stress usually results in a cell membrane injury and damage to macromolecules and organelles leading to cell death, the activation of antioxidant adaptive response at a moderate level of oxidative stress is well recognized phenomenon. This diverse cellular response is connected with activation of different cellular signalling pathways, such as MAP kinases cascade and/or pathways associated with redox-sensitive transcription factors, such as HIF-1, NF-κB and Nrf-2 [26–28] The activation of NF-κB transcription factor and an increased expression of its target genes was also reported in HepG2 cells treated with AgNPs [29]. In addition, adaptation of HepG2 cells to AgNPs induced oxidative stress has been already proposed as a mechanism explaining the differences in response to nanosilver between HepG2 and A549 cell lines [30]. In that work a different AgNPs susceptibility of HepG2 and A549 cells was linked to upregulation of pro-proliferative and anti-apoptotic signalling pathways.

In our experimental setup, an observed increase of antioxidant enzymes activities resulted as an adaptation to low glucose condition and accompanied increase of oxidative stress. The impact of glucose on cell metabolism has been widely studied and there are numerous data on the effects of glucose availability on oxidative stress induction. High glucose concentration leads to increased protein glycation, but provides more sustainable environment for cell growth. However, increased mitochondrial protein glycation and accumulation of advanced glycation end-products may lead to mitochondrial dysfunction and oxidative stress, as has been shown on *C. elegans* [31] and human cells [32].

On the other hand, ROS generation has been also associated with low glucose availability [33], which is consistent with reports of switching cell metabolism from glycolysis to OXPHOS [10, 34]. In physiological conditions the prevalent way for energy supply in cancer cells is glycolysis, thus glucose scarcity forces the metabolic switch back to OXPHOS (so-called the Warburg effect) [35]. The phenomenon of metabolic switch between glycolysis and respiratory chain in response to glucose availability was observed in a variety of experimental setups, ranging from yeast to mammalian cell lines [36, 37]. The Warburg effect was firstly explained by irreversible damage to the elements of oxygen-dependent pathway of OXPHOS in cancer cells. However, this explanation was questioned by the recent investigations showing an intact functionality of mitochondrial OXPHOS in many cancer cells [38–40] and the studies describing similar effect in non-cancer, proliferating cells, which were not supposed to have the OXPHOS pathway irreversibly damaged [41]. Moreover, many authors consider the Warburg effect as a result of suppression of mitochondrial OXPHOS due to enhanced glycolysis rather than defects in its functionality. If glycolysis is inhibited in cancer cells, the function of mitochondrial OXPHOS can be restored [38, 42, 43]. Indeed, also in our experimental setup, depletion of glucose supply resulted in metabolic switch and enhanced production of H_2O_2 in mitochondria due to the OXPHOS. As oxidative stress prolonged, cells adopted to the new situation by elevating the activity of key antioxidant defence enzymes (Table 4).

This was further confirmed by the transcriptome analysis. The level of catalase gene mRNA (*CAT*) was elevated 3.5-times in cells cultured in the low glucose medium, as compared to cells cultured in high glucose medium. Catalase is a key peroxisome antioxidant enzyme detoxifying H_2O_2 and plays a protective role against H_2O_2-induced oxidation in nearly all aerobic organisms. A similar increase of the *CAT* expression was observed in response to oxidative stress [44] and low glucose condition [45]. Glucose scarcity resulted also in upregulation of the Scavenger Receptor Class A, Member 3 (*SCARA3*) mRNA level (3.5 times). Being a macrophage scavenger receptor-like protein, the Scara3 protein has been shown to deplete ROS and other harmful products of oxidation, thus playing an important role in protecting cells from oxidative stress. Brown et al. [46] reported an increase of SCARA3 expression in cells treated with H_2O_2.

Of note is the 50-times increase of mRNA level of *GSTM5* gene that belongs to the mu class of glutathione *S*-transferases (Table 3), although accompanied only by 1.7 times higher activity of the enzyme (Table 4). The Gstm5 protein functions in detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with reduced glutathione. Overexpression of *GSTM5* has been already reported due to the generation of ROS in low-glucose medium [47]. A similar phenomenon was found in AgNPs treated *Arabidopsis thaliana* roots [48] that suggest a common mechanisms of response to the increased mitochondrial generation of ROS. A marked up-regulation of expression was also observed for albumin gene (*ALB*). Albumin is a liver-derived, the most abundant plasma protein well recognized for its antioxidant properties [49, 50], Human serum albumin was shown to prevent neuronal death in murine cortical cell cultures exposed to oxidative stress generated by H_2O_2 [51].

In addition to antioxidant proteins and enzymes, another markedly up-regulated gene was a Mannose-Binding Lectin (Protein C) 2, Soluble (*MBL2)*, which plays an important role in innate immune defence system. Another important function of MBL2 protein is binding to the apoptotic and necrotic cells that facilitate their uptake by macrophages. MBL2 is produced in the liver in a response to infection, typically associated with generation of oxidative stress [52].

Noteworthy, although Zn-Cu superoxide dismutase activity was still elevated, transcritpome analysis revealed that its mRNA transcription was already shut down (Tables 3, 4). This discrepancy can be easily explained by increased activity of mitochondrial respiratory chain induced by low glucose condition and mutually associated increase of its unwanted by-product, superoxide anion radical. Moreover, the Neutrophil Cytosol Factor 1 (*NCF1*) mRNA level was increased 2.9 times. NCF1 protein is a subunit of NADPH oxidase, the enzyme also producing superoxide anion radical. Superoxide anion radical is subsequently converted into H_2O_2 by superoxide dismutase. As superoxide anion radical is less reactive than H_2O_2-derived hydroxyl radical and due to the negative charge does not easily cross the cell membranes, it is probably less dangerous and more favourable as an excess, resident ROS than H_2O_2. In agreement, catalase activity and transcription was elevated (Tables 3, 4).

Surprisingly, low glucose concentration in culture medium resulted in down-regulation of several genes involved in synthesis and metabolism of glutathione (*GSS, GSTZ1, GSTA4, GPX*), This suggest that general detoxification of ROS generated in low glucose condition is carried out with fast enzymatic reactions rather than small molecule antioxidants, such as glutathione. Down-regulation of glutathione synthesis is in line with up-regulation of glutathione S-transferase, that deplete the reduced glutathione by conjugating with nucleophiles, the conjugates are further removed by ABC-type transporters [53]. Since glutathione may act as an anti- and pro-oxidant [54], it seems favourable to diminish glutathione redox-cycling mechanism in chronic oxidative stress condition. Moreover, in chronic oxidative stress GSH depletion may promote pro-survival pathways. Whereas, a rapid GSH depletion induces pro-apoptotic pathway [55], its slow and prolonged depletion triggers NFkB signalling pathway and associated anti-oxidative and pro-survival response [56]. Activation of NFkB signalling pathway is repeatedly reported in oxidative stress condition of different origin, including AgNPs treatment [29]. However, it must be remembered that different signalling pathways often stimulated by the same condition (e.g. oxidative stress) may interact each other, as recently reviewed for NFkB and NRF/KEAP signalling pathways [57] and many others [58] or even counteract, e.g. mitigation of oxidative stress induced NFkB pathway activity by HIF-1 [59]. Thus, a net effect of signalling pathways stimulation is a result of pathways crosstalk and local, intracellular context.

Conclusions

We have shown that glucose availability can modify toxicity of AgNPs via elevation of antioxidant defence triggered by oxidative stress resulted from enhanced OXPHOS in mitochonddria and associated generation of ROS. Since the mechanism does not depend on the toxic factor but the activity of mitochondria, it seems to be universal, regarding not only AgNPs, but all oxidative stress generating agents, e.g. another nanomaterials, pesticides and many others. Moreover, our results suggest that cells relying strongly on glycolysis (such as cancer cells) might be more prone to toxic action of nanomaterials that normal cells, whose metabolism is based on the OXPHOS. Further, higher survival of cells cultured in environment forcing the activation of mitochondrial respiratory chain, as compared with glycolysis based ones, gives a good prognosis for use of nanomaterials in medical and pharmaceutical application.

On the other hand, few crucial cell types in the human body strongly rely on glycolysis, including brain, muscle and liver cells [60] that makes them more susceptible to the toxic action of nanomaterials than OXPHOS based cells, unless have other mechanisms elevating antioxidant response.

Finally, our results suggest that local microenvironment, such as local availability of glucose, might be a factor promoting an adaptive abilities of cells, and modifying their response to xenobiotics, including nanomaterials.

Methods

Cell culture and treatment

HepG2 cell line was purchased from the American Type Culture Collection (ATCC). The cells were cultured in 75 cm^3 Nunclon flasks in glucose supplemented Dulbecco's Modified Eagle Medium (DMEM). Medium described in this paper as a high-glucose medium contained glucose at the concentration of 4.5 g/dm^3 (25 mM), whereas low glucose medium contained 1 g/dm^3 glucose (5.5 mM). Both variants of the medium were supplemented with 10 % FCS (Gibco). Glucose concentrations roughly reflect average blood sugar level and heavy hyperglycaemic level [61]. Cells were maintained in a 5 % CO_2 atmosphere at 37 °C at 95 % relative humidity. Cells described further as being cultured for long period of time on low glucose were passaged for 30 days before experiments in medium with 5.5 mM glucose.

Cells referred to as sustained for short period of time on low glucose were incubated in medium with 5.5 mM glucose for 48 h, including AgNPs treatment.

Silver nanoparticle preparation

The stock suspension (2 mg/cm^3) of the AgNPs of a 20 nm nominal diameter (PlasmaChem, Germany) was prepared as previously described [13]. In brief, AgNPs were triturated in agate mortar, then 2 mg of NPs was suspended in 800 µL of water. Suspension was sonicated (4.2 kJ/cm^3) with OmniRuptor 4000 and then 100 µL of 15 % albumin which was followed by addition of 100 µL of tenfold concentrated PBS (1.37 M NaCl, 27 mM KCl, 80 mM Na_2HPO_4, 20 mM KH_2PO_4). Intermediate dilutions were made in DMEM with the corresponding high or low glucose content.

Nanoparticles' hydrodynamic diameter and zeta potential was measured by dynamic light scattering (DLS, Malvern, UK). Both the hydrodynamic diameter and zeta potential of the samples were measured at 25 °C with a scattering angle of 173°. The particle concentration for hydrodynamic diameter measurements in each sample was 2 mg/cm^3 in the preparation buffer. Measurements were done in triplicate with over 14 sub-runs. The pH value of the suspensions was 7.1.

A detailed characterization of this batch of AgNPs, including different preparation protocols, dispersion, aggregation and stability in different culture media, as well as silver ion release, was previously described in [21, 62]

Measurement of H_2O_2 generation in mitochondria

HepG2 cells were suspended at a density of 1×10^6 cells/cm^3 and centrifuged for 10 min at 200×*g* in 1.5 cm^3 Eppendorf tube. The supernatant was removed, the cells were resuspended in 0.8 cm^3 of hypoosmolar buffer (Eppendorf cat no 940002001), and 10 µg of HyPer-mito plasmid (EVROGEN, Russia, cat.# FP942) was added. Cells were transferred to a cuvette and electroporated with three 10 µs pulses with a voltage of 500 V (Multiporator®, Eppendorf).

After electroporation cells were plated on 6-well plate (Nunclon) in 3 cm^3 of DMEM medium with an appropriate glucose concentration (5.5 or 25 mM). After 24 h of culture cells were trypsinized with 0.25 cm^3 of 0.25 % trypsin, suspended in 0.75 cm^3 of appropriate DMEM medium and transferred to cytometry tubes. Fluorescence intensity of transfected cells was measured using a LSRII flow cytometer in the FITC channel. Transfection efficiency was found to be at the level of 60–70 %. For further analysis only cells that exhibited fluorescence above the maximum autofluorescence of mock transfected cells were used.

To check the approximate range of H_2O_2 detection by the product of the reporter vector, HyPer-mito plasmid transfected HepG2 cells were cultured in 25 mM glucose and titrated with extracellular H_2O_2. Five minutes after H_2O_2 addition cells' fluorescence was read in a LSRII cytometer.

Neutral red viability test

Cells were plated on 96-well plates (Nunclon) at a density of 15 000 cells per well in a final volume of 0.1 cm^3. After 24 h, an appropriate aliquot of AgNPs was added to achieve concentrations in range from 2.5 to 50 µg/cm^3 in a final volume of 0.2 cm^3 After next 24 h incubation, the medium was removed and cells were washed twice with 0.15 cm^3 per well of PBS solution. The cells were then flooded with 0.1 cm^3 of neutral red solution (50 µg/cm^3 neutral red in the culture medium). After a 4-h incubation at 37 °C in an atmosphere of 5 % CO_2 neutral red solution was discarded, the cells washed twice with 0.15 cm^3 PBS and 0.15 cm^3 of fixative (50 % of 96 % ethanol, 49 % H_2O, 1 % acetic acid) was added to each well. The plate was shaken for 15 min and the absorbance was measured at a wavelength of 540 nm using an EnVision® Multilabel Reader (Perkin-Elmer).

Gene expression analysis

Total RNA was isolated from 10^6 HepG2 cells employing MagNA Pure LC 2.0 Instrument (Roche) according to the manufacturer's protocol. Genomic DNA was removed by DNase I digestion (RNase free DNAse, Life Technologies) and 1 µg of the total RNA was reverse-transcribed using the SuperScript™ III First-Strand Synthesis SuperMix (Life Technologies). qPCR analysis was performed with C1000 Thermal Cycler–CFX96 Real-Time System (BioRad).

The human oxidative stress library (HOSL-1) primers (RealTimePrimers.com) were used to assess the expression of oxidative stress related genes using iQ SYBR Green Supermix (BioRad). After 3 min of an initial activation and denaturation step at 95 °C, 40 cycles of denaturation at 95 °C for 10 s, annealing/extension at 60 °C for 45 s followed by melt curve analysis (55–95 °C, 0.5 °C increment, 5 s/step) were performed. cDNA was omitted in non-template control. The mRNA level was calculated using the ΔΔct method (Rest, Qiagen), and normalized to the housekeeping gene (*ACTB),* as a control.

Assessment of antioxidant enzymes activity in cells homogenate

For biochemical analysis cells were plated in 75 cm^3 Nunc flasks, 3×10^6 cells per flask and cultured in DMEM with low or high glucose concentration at 5 % CO_2 atmosphere, 37 °C and 95 % relative humidity. For enzymatic

activity estimation, the cells were harvested by trypsinization when cultures reached 80 % confluence (roughly after 48 h culture), lysed with 0.1 % Triton/1 mM EDTA solution and frozen overnight at −20 °C. All measurements were conducted in 96-well NUNC plates in an EON spectrophotometer (BioTek).

Catalase activity of samples was determined by assessing their ability to decompose H_2O_2 as previously described [63]. Gluthatione S-transferase activity was determined with colorimetric method as described by Rise-Evans et al. [64]. SOD activity was assessed by the NBT reduction method [65]. Glutathione reductase was assayed on the basis of NADPH oxidation [66]. The results were referenced to protein concentration in the samples and enzymatic activity was calculated by comparison in comprehension with enzyme standards. All methods were adapted to 96-well microplates by scaling down the volume of reagents.

Statistical evaluation

All statistical evaluations were made using a GraphPad Prism 5, Microsoft Excel and REST software. Curves graphed in Fig. 1 were compared utilizing extra sum of square F test. Difference between various inhibitory concentrations summarized in Table 2 were calculated by ANOVA with Tukey`s post hoc test. Statistical significance of differences in generation of hydrogen peroxide induced by different treatments, summarized in Fig. 3 was calculated with ANOVA followed by Tukey`s post hoc test. The expression ratio results (Table 3) of the investigated transcripts were tested for significance by a Pair Wise Fixed Reallocation Randomisation Test and error is expressed as a standard error (SE) estimated via Taylor algorithm (REST software) [67]. Enzymes activities summarised in Table 4 were compared with t test.

Additional file

Additional file 1. Expression on mRNA level of all tested genes. Results are shown as fold change of expression in cells sustained on low glucose medium (5.5 mM) when compared with cells sustained on high glucose medium (25 mM).

Abbreviations

AgNPs: silver nanoparticles; DLS: dynamic light scattering; OXPHOS: oxidative phosphorylation; ROS: reactive oxygen species; HIF-1: hypoxia inducible factor 1; NF-κB: nuclear factor kappa B; Nrf-2: nuclear factor (erythroid-derived 2)-like 2 (NFE2L2).

Authors' contributions

MZ: carried out survivability assays, mitochondrial hydrogen peroxide assessment, assessment of enzymatic activities, statistical evaluation of data, preparation of manuscript text. DW: carried out preliminary survivability assays, mitochondrial hydrogen peroxide assessment. DK: carried out RT–PCR gene expression profiling. SMW: carried out nanoparticle characterization. MK: have made substantial contributions to conception and design of the study, drafted and critically revised the manuscript and approved its final version for submission. AG: have made substantial contributions to conception and design of the study, drafted and critically revised the manuscript and approved its final version for submission. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Research Paper

Silver nanoparticles can attenuate nitrative stress

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ABSTRACT

We have reported previously that glucose availability can modify toxicity of silver nanoparticles (AgNPs) via elevation of antioxidant defence triggered by increased mitochondrial generation of reactive oxygen species. In this study, we examined the effect of glucose availability on the production of reactive nitrogen species in HepG2 cells and modification of nitrative stress by AgNPs. We found that lowering the glucose concentration increased expression of genes coding for inducible nitric oxide syntheas, NOS2 and *NOS2A* resulting in enhanced production of nitric oxide. Surprisingly, AgNPs decreased the level of nitric oxide accelerated denitration of proteins nitrated by exogenous peroxynitrite in cells grown in the presence of lowered glucose concentration, apparently due to further induction of protective proteins.

1. Introduction

In recent years interest in silver nanoparticles and their applications has increased mainly because of the important antimicrobial activities of these nanomaterials, allowing their use in several industrial sectors. These broad applications, however, increase human exposure and thus the potential risk related to their short- and long-term toxicity. There is increasing concern with respect to the biological impacts of the use of silver nanoparticles on a large scale, and the possible risks to the health and environment [1]. A large number of in vitro studies indicate that AgNPs are toxic to the mammalian cells derived from skin, liver, lung, brain, vascular system and reproductive organs. Interestingly, some studies have shown that these particles have the potential to induce genes associated with cell cycle progression, DNA damage and apoptosis in human cells even at non-cytotoxic doses [2].

An important mode of action of AgNPs is the induction of oxidative stress. Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses or, more recently, as disruption of redox signaling and control [3]. Numerous data exist in the literature that both ROS and reactive nitrogen species (RNS) are produced in a well-regulated manner to help maintain homeostasis at the cellular level in the normal healthy tissues, play an important role as second messengers, and regulate cellular function by modulating signaling pathways [4]. However, excessive production of ROS and RNS results in damage to all components of the cell, including proteins, lipids, and DNA.

Stress induced by reactive nitrogen species (RNS) is referred to as nitrative (nitrosative) stress. Nitric oxide (NO), endogenous precursor of RNS, is produced mainly by nitric oxide synthases (NOS) in various cell types such as endothelial cells [5], liver cells [6] and macrophages [7]. It participates in cellular signaling [8] and controls the activities of several enzymes, first of all guanyl cyclase [9]. At the cellular level, NO affects cell apoptosis and participates in immune defense [10] while at the organismal level its main function is the control of blood pressure [11].

While NO shows a limited reactivity, a product of its reaction with the superoxide radical anion, peroxynitrite $ONOO^-$, is a reactive oxidant and nitrating agent. The reaction of peroxynitrite formation is characterized by a very high rate constant, of about $1.6*10^{10}$ [12] so it proceeds *in vivo* even though the concentrations of both substrates are low and is enhanced when production of the precursors is increased or when the activity of superoxide dismutase is compromised (which occurs in many pathologies). Peroxynitrite is unstable under physiological conditions, one way of its decomposition leading to the formation of the hydroxyl radical and nitrogen dioxide [13]. It is therefore a strong oxidant and nitrating agent modifying proteins, lipids and nucleic acids and depleting cellular antioxidants. Proteins are subject to various modifications by peroxynitrite, the most typical modification being nitration of tyrosine residues [14,15].

Oxidative stress induced by engineered NP is due to acellular factors such as particle surface, size, composition, and presence of metals, while cellular responses include mitochondrial respiration, NP-cell interaction, and immune cell activation are responsible for ROS-

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mediated damage. NP-induced oxidative stress responses are torch bearers for further pathophysiological effects including genotoxicity, inflammation, and fibrosis as demonstrated by activation of associated cell signaling pathways [16]. Various factor, among them glucose availability, modulate AgNPs-induced oxidative stress [17] or the presence of other xenobiotics. The main cellular source of superoxide is the respiratory chain in the mitochondria [18]. If oxidative stress caused by increased activity of the respiratory chain is of moderate intensity, the cell can adapt to its occurrence [17], which results in an increased resistance to oxidative stress induced by other agents, e. g. AgNPs. Adaptation is mainly due to activation of the Nrf2 pathway [19], NF-κB [20] or the MAPK pathway [21]. Metabolic pathways regulated by oxidative stress control the level of many proteins responsible for the cellular redox balance.

In this paper, we have studied how AgNPs can modulate nitrative stress induced by increased production of reactive oxygen species in mitochondria and increased activity of iNOS in HepG2 cells.

2. Materials and methods

2.1. Cell culture

HepG2 cell line was derived from hepatocellular carcinoma of a Caucasian male adolescent. HepG2 cells can be cultured in media containing different concentrations of glucose [22] and are a well recognized model for nanotoxicity testing. HepG2 cell line originated from the American Type Culture Collection (ATCC). The cells were cultured in Dublecco's Modified Eagle Medium (DMEM) supplemented with FBS (Gibco). Cells were maintained in a 5% CO_2 atmosphere at 37 °C, at 95% relative humidity. During passages cells were detached from culture surface with trypsin solution (0.25 trypsin, 1 mM EDTA in PBS, 10 min incubation in culture conditions).

2.2. Neutral red viability assay

Cells were plated on 96-well plates (Nunclon) at a density of 15,000 cells per well in a final volume of 0.1 cm^3. After 24 h, an appropriate aliquot of AgNPs was added to achieve concentrations in range from 2.5 to 50 μg/cm^3 in a final volume of 0.2 cm^3. The procedure of preparation of nanoparticles is as described by Zuberek at al. [17]. After next 24 h incubation, the medium was removed and cells were washed twice with 0.15 cm^3 per well of PBS solution. The cells were then flooded with 0.1 cm^3 of neutral red solution (50 μg/cm^3 neutral red in the culture medium). After a 4h incubation at 37 °C in an atmosphere of 5% CO_2 neutral red solution was discarded, the cells washed twice with 0.15 cm^3 PBS and 0.15 cm^3 of fixative (50% ethanol, 49% H_2O, 1% acetic acid) was added to each well. The plate was shaken for 15 min and the absorbance was measured at a wavelength of 540 nm using an EnVision® Multilabel Reader (Perkin-Elmer) [23].

2.3. Gene expression analysis

Total RNA was isolated from 10^6 HepG2 cells employing MagNA Pure LC 2.0 Instrument (Roche) according to the manufacturer's protocol. Genomic DNA was removed by DNase I digestion (RNase free DNase, Life Technologies) and 1 μg of the total RNA was reverse-transcribed using the SuperScript™ III First-Strand Synthesis SuperMix (Life Technologies). qPCR analysis was performed with C1000 Thermal Cycler–CFX96 Real-Time System (BioRad). Primers were obtained from RealTimePrimers.

2.4. Nitric oxide measurement

Cells for flow cytometry evaluation of nitric oxide were plated on 6-well plates in density of 167·10^5 cells/cm^3 in 3 cm^3 of low or high glucose DMEM. AgNPs were added after 24 h to final concentration of 25 μg/ml for another 24, 48 or 72 h of incubation, after which cells were trypsinized and resuspended in 1 cm^3 of culture medium. DAF-FM was added to final concentration of 5 μM and fluorescence intensity was recorded in FITC channel (LSRII flow cytometer) after 30 min of incubation in 37 °C.

2.5. Measurement of reactive oxygen species

Cells for flow cytometry evaluation of nitric oxide were plated on 6-well plates in density of 167·10^5 cells/cm^3 in 3 cm^3 of low or high glucose DMEM. AgNPs were added after 24 h to final concentration of 25 μg/ml for another 4 h of incubation, after which cells were trypsinized and resuspended in 500 mm^3 of full growth media. After 10 min in 37 °C 100 mm^3 of DMEM solution of appropriate probe was added to cell suspension to form final concentration of 5 μM for another 45 min of incubation. Cellular levels of hydrogen peroxide, cellular superoxide anion and mitochondrial superoxide anion was measured respectively with DHR123, DHE and MitoSOX.

2.6. Measurement of nitrotyrosine in HepG2 cells

Cell cultures grown in 6-well nunclon delta plates at density of 150,000 cells/cm^3 were incubated for 5 min with peroxynitrite in concentration of 1 mmol/dm^3 after 2 h of incubation with AgNPs (25 μg/ml), after peroxynitrite treatment monolayer was washed twice with PBS. Cells were lysed and scrapped in RIPA buffer immediately after peroxynitrite incubation and after another 2 or 4 h of incubation in culture conditions.

2.7. Measurement of nitrotyrosine

Tyrosine in concentration of 1 mmol/dm^3 was nitrated with 1.4 mmol/dm^3 peroxynitrite in presence of AgNPs in concentration of 12.5 μg/cm^3 or in the presence of BSA in concentration corresponding to one used in preparation of AgNPs suspension. Fluorescent spectra of tyrosine were obtained at 315 nm excitation wavelength.

3. Results

3.1. Cell survival

Comparison of the effects of AgNPs on cell survival showed that cells grown in 25 mM glucose and transferred to medium of a lower glucose concentration (5.5 mM) for 48 h were more resistant than cells maintained in 25 mM glucose (Fig. 1). This result confirms our previous observations on the higher toxicity of AgNPS to HepG2 cells grown on high glucose as compared with cells cultivated on low glucose for a prolonged time [17].

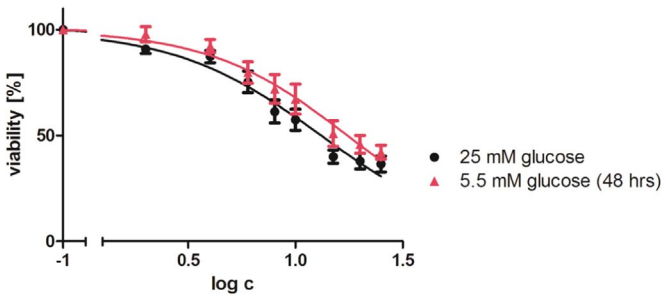

Fig. 1. Effect of AgNPs treatment on the survival of HepG2. Cells were grown in high glucose DMEM and transferred to either high glucose DMEM (25 mmol/dm^3) or low glucose DMEM (5.5 mmol/dm^3). After 48 h, cells were treated with AgNPs (2.5–50 μg/cm3). After 24 h of incubation viability was assessed with neutral red method. Curves were compared with extra sum of square F test and are statistically different, p=0.0118, N=12 (high glucose), N=9 (low glucose).

Table 1
Expression of inducible nitric oxide synthase and NADPH oxidase on mRNA level in HepG2 cells. Cells were grown on low glucose DMEM for 72 h prior to RNA isolation. Expression is presented as fold change of expression in cells cultured on low glucose (5.5 mmol/dm^3) relative to cells cultured on high glucose (25 mmol/dm^3). Significance was tested via Pair Wise Fixed Reallocation Randomisation Test using REST 2009 software.

Gene	Expression	95% C.I.	Result
NOS2	**27.284**	23.721–29.651	**UP**
NOS2A	**25.516**	22.534–27.974	**UP**
NOX5	**1.886**	0.524–4.254	–

3.2. Analysis of gene expression

Lowering glucose concentration in the culture medium increased the mRNA level for NOS2 and NOS2A. No significant changes were found for NADPH oxidase 5, the main extramitochondrial source of superoxide radical (Table 1).

3.3. Nitric oxide level

Decrease of glucose concentration from 25 down to 5.5 mol/dm^3 lead to stabilization of intracellular nitric oxide concentration at higher level after 72 h but not at 24 or 48 h (Fig. 2). This level was not different from that found in cells grown on low-glucose medium for a month or longer. These results demonstrate that a new redox balance is attained in the cells after 3 days, apparently being an element of cellular adaptation to augmented oxidative stress caused by lowered glucose concentration.

Comparison of nitric oxide levels in cells grown on different glucose concentrations revealed that AgNPs did not induce any detectable increase in nitric oxide in cells grown in 25 mM glucose and even decreased nitric oxide generation in cells grown in 5.5 mmol/dm^3 glucose (Fig. 3).

3.4. Production of reactive oxygen species

Cystosolic superoxide level was considerably lower in cells grown in high glucose than in cells grown in low glucose. AgNPs did not affect significantly the cytosolic superoxide level in cells grown in both high and low glucose concentrations (Fig. 4A). Mitochondrial superoxide level measured with MitoSOX was also lower in cells grown in low glucose (as compared with cells grown in high glucose); AgNPs increased superoxide level in cells grown in high glucose while not affecting superoxide production in cells grown in low glucose (Fig. 4C). The cellular level of peroxides reflected that of mitochondrial superoxide (Fig. 4B), confirming that mitochondria were the main source of cellular peroxides in HepG2 cells.

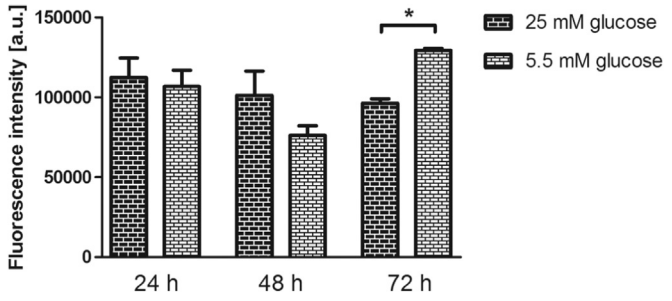

Fig. 2. The effect of high glucose medium on the nitric oxide level in HepG2 cells. The cells were grown in high glucose medium. Then the cells were transferred either to a low glucose (5.5 mol/dm^3) or high glucose medium (25 mol/dm^3). The level of nitric oxide was determined after 24 h, 48 h and 72 h with 5 μM DAF-FM in a fow cytometer. Asterisk denotes statistically significant difference ($P < 0.05$). Bars represent means and error bars represent SEM.

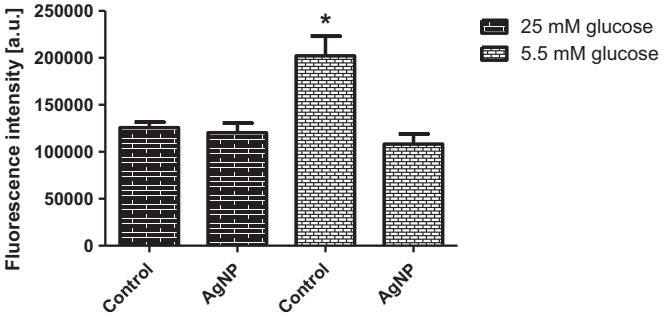

Fig. 3. The level of nitric oxide in HepG2 cells treated with AgNPs. The cells were grown in DMEM medium containing low (5.5 mmol/dm^3) or high (25 mmol/dm^3) glucose concentration. The cells were treated with AgNPs (25 μg/cm^3) for two hours. Then the nitric oxide in the cells was estimated with 5 μM DAF-FM in a flow cytometer.r Asterisk denotes statistically important difference between cells cultured on low glucose concentration (5.5 mmol/dm^3) and all other samples ($P < 0.05$). Bars represent means and error bars represent SEM.

3.5. Nitrotyrosine level

Endogenous level of protein nitration in HepG2 cells was below the detection level of the ELISA test used. Protein nitration induced by exogenous peroxynitrite was higher in cells grown in lowglucose medium. AgNPs treatment tended to increase the level of protein nitration in cells grown on high glucose (not reaching statistical significance) and did not affect the level of protein nitration in cells grown on low glucose. The level of nitrotyrosine decreased during subsequent incubation, the rate of denitration being lower in cells grown in 5.5 mM glucose. After 2–4 h the level of nitrotyrosine dropped down below the detection limit. In cells grown on low glucose, the rate of nitrotyrosine removal was higher in the presence of AgNPs (Fig. 5).

Control experiments in which tyrosine nitration was determined fluorimetrically demonstrated the AgNPs inhibited nitration of tyrosine (Fig. 6).

4. Discussion

Our previous paper demonstrated that glucose availability can modulate the toxic action of AgNPs via regulation of levels of proteins involved in cellular antioxidant defense [17].

We have found an increased activity of mitochondria, responsible for oxidative phosphorylation, under lowered glucose conditions. This effect activates signaling pathways controlled by cellular levels of ROS. We expected that stress induced by lowered glucose will be augmented by stress induced by nanoparticles. To the contrary, the cells cultured after prolonged (over one month) under low glucose conditions were found to be less susceptible to AgNPs than cells grown in 25 mM glucose. We ascribe this effect to the stimulation of antioxidant defense in cells stressed by low glucose conditions, as evidenced, i. a., by increased activities of main antioxidant enzymes [17]. This process of adaptation requires time and first discernible effects can be observed at 48 h after the change of the medium.

Induction of oxidative stress induced by transfer of the cells to increased glucose concentration has been reported in the literature. This effect is ascribed to the production of free radicals in the process of glucose autoxidation. Glucose can enolize and form ROS (superoxide anion and hydrogen peroxide) even under physiological conditions [24]. Increased generation of ROS may slow down the rate of cell proliferation [25]. However, some authors reported induction of ROS generation by cell transfer to medium containing low glucose concentration. This effect took place already 15–30 min after the lowering of glucose concentration in the medium and was inhibited by rotenone, which points to Complex I of the respiratory chain as the main source of ROS [26].

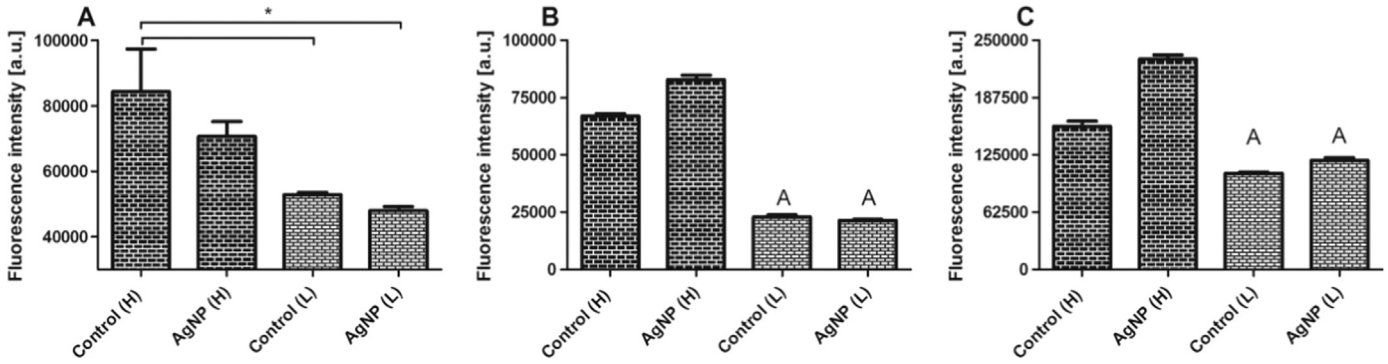

Fig. 4. Levels of cellular superoxide anion (A), cellular peroxides (B) and mitochondrial superoxide anion (C) measured with DHE, DHR123 and MitoSOX respectively in HepG2 cells cultured on two different concentrations of glucose (H – 25 mmol/dm^3, L – 5.5 mmol/dm^3) and treated with AgNPs at a concentration of 25 μg/cm^3. Bars represent means and error bars represent SEM. Asterisk in panel A represent statistically significant difference (α=0.05, n=3). Capital letters A in panels B and C denote groups between which we found no statistically significant difference; otherwise the differences are statistically significant (α=0.05, n=3(B), n=6(C), n=6(D)). Groups in presented experiments were compared with ANOVA followed by Tukey's post hoc test.

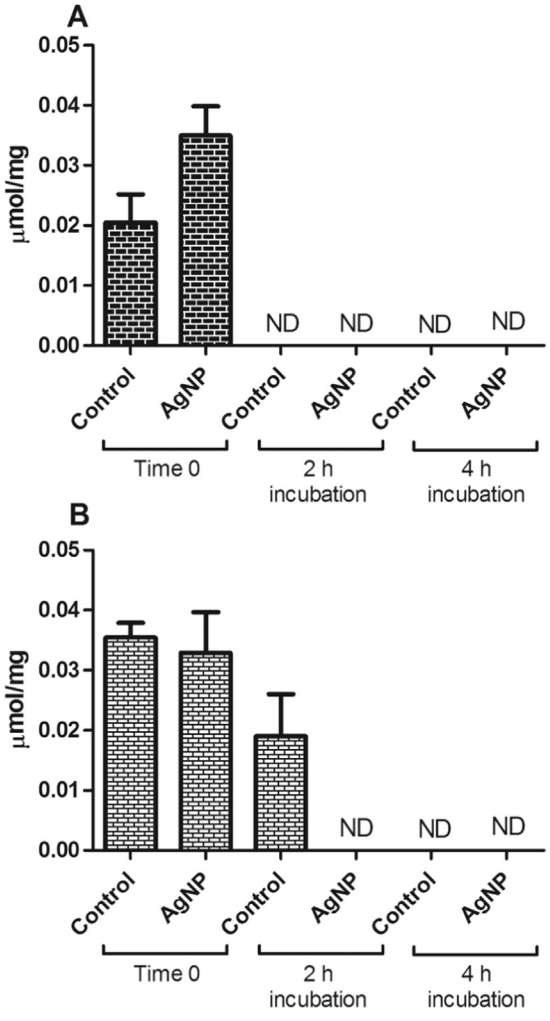

Fig. 5. Nitrotyrosine level in proteins from HepG2 cell lysates cultured in high glucose (25 mmol/dm^3) (A) or low glucose (5.5 mmol/dm^3) (B) DMEM. Cell cultures were incubated for 5 min with peroxynitrite in concentration of 1 mmol/dm^3 in 1 cm^3 of PBS solution, after 2 h of incubation with AgNPs (25 μg/cm^3). Cells were lysed immediately after peroxynitrite incubation and after 2 and 4 h. Bars represent means and error bars represent SEM (n=3), ND stands for not detectable.

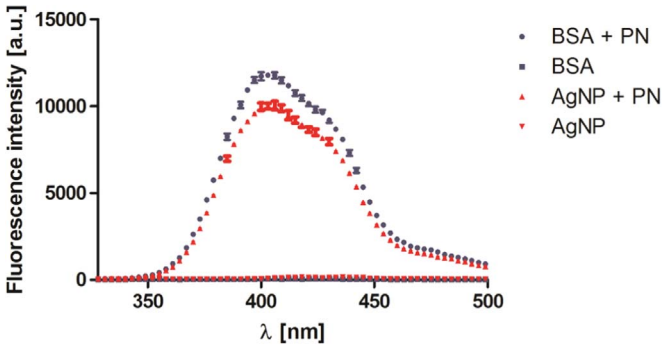

Fig. 6. Effect of AgNPs on the nitration of tyrosine (TYR) by peroxynitrite. TYR (1 mmol/dm^3 final concentration) in PBS was mixed with peroxynitrite (1.4 mmol/dm^3 final concentration) in the presence of AgNPs in concentration 12.5 μg/cm^3 or in the presence of BSA in concentration corresponding to one used in preparation of AgNPs suspension. Fluorescence spectra (α_{ex}=315 nm) of nitrotyrosine were measured in a EnVision® Multilabel Reader.

In this paper, we used HepG2 as a model cell line. This line adapts easily to various culture conditions [22]. ATCC recommends culturing these cells in medium containing 1 g/L of glucose but these cells grow well in a medium containing 25 mM glucose, and only 50 mM glucose limits cell survival and induces apoptosis after prolonged incubation (72 h) [27]. HepG2 cells are derived from hepatocytes, in which glucose conditions may be high due to glycogenolysis and gluconeogenesis as these cells produce glucose and release it to the bloodstream so high glucose conditions may imitate better the *in vivo* conditions for these cells [28].

Analysis of survival data and expression of oxidative stress-related genes [17] allows to conclude that adaptation of HepG2 cells to new culture conditions requires at least 72 h. A significant increase in the level of transcript coding inducible NOS has been observed not earlier but after 72 h of culture in lowglucose medium. This enzyme is responsible for generation of relatively high concentrations of nitric oxide in the cells. The increased activity of iNOS is maintained during at least one-month culture of the cells in low glucose (not shown). Apparently, this stimulation of iNOS expression may be due to disturbances in the redox balance caused by increased activity of the mitochondrial respiratory chain.

Transcriptional regulation of the gene responsible for the production of iNOS is complex and still not fully understood. Several signaling cascades have been found to control the expression of iNOS in cells at the transcriptional level, including JNK/ p38, NF-κB, JAK and STAT [29]. The iNOS promotor is controlled by numerous transcription factors, the most important being NF-κB p50: p65. This factor is activated, i. a., by lipopolysaccharide (LPS), viral products, cytokines, cell–cell contact, neurotoxins and oxidative stress [6]. The C/EBP transcription factor, activated by LPS, viral products, CD40 ligation, cAMP, hypoxia IL-1β, dsRNA, glucose metabolites acts probably as a co-activator [30]. The STAT-1 transcription factor has been identified

as a negative regulator of the iNOS gene [31,32]. The role of AP1 proteins in the regulation of iNOS promotor is still controversial; it was found, however, that Fra-1, Fra-2, JunB, JunD, and FosB transcription factors are involved in the regulation of iNOS expression and NO production [33,34]. On this basis it can be expected that ROS play a key role in the regulation of iNOS expression and a disturbance in the redox balance may lead to an increased NO generation in the cells.

We observed decreased levels of cytoplasmic superoxide and hydrogen peroxide in cells grown on low glucose upon incubation with AgNPs (Fig. 4). Increased ROS generation by nanoparticles has been reported by many authors. However, increased generation of hydrogen peroxide in cells treated with AgNPs is usually found in high-glucose media (or precise information on culture conditions is lacking) and the studies do not consider the endogenous ROS production by mitochondria and its effect on the redox state of the cells.

This effect may not reflect the true level of superoxide but to be due to the lower accessibility of superoxide for the fluorogenic probe. Superoxide reacts rapidly with NO forming peroxynitrite. This reaction proceeds at a very high rate and is diffusion-controlled ($k \sim 1\times10^{10}$ M^{-1} s^{-1}). The reaction rate constant of superoxide with dihydroethidine is much lower ($2.17 \pm 0.059\times10^{3}$ M^{-1} s^{-1}), so in spite of the higher concentration of the probe with respect to NO, the latter may compete significantly for superoxide, the more that it is constantly produces in the system [35]. The mitochondrial production of superoxide was increased in cells grown on low glucose (Fig. 4), which may underlie this effect. Similarly, the level of NO was decreased in cells grown in low glucose by treatment of AgNPs. Both these observations are compatible with the hypothesis that increased consumption of superoxide and NO can explain the lower level of superoxide, hydrogen peroxide and NO found in cells grown on low glucose, inducing higher iNOS expression. Alternatively, it can be suggested that AgNPs increase biosynthesis of antioxidant proteins which decompose ROS and inhibit the activity or attenuate the expression of iNOS, especially under low glucose conditions.

We found increased protein nitration in cells grown on low glucose by exogenous peroxynitrite. Nitrotyrosine formation is one of the main protein modifications induced by peroxynitrite. The level of protein nitration by endogenously produced peroxynitrite is low due to the efficient protection against peroxynitrite and protein denitratase activity. Indeed, low levels of peroxynitrite could be detoxified by enzymatic and nonenzymatic systems [36–39]. In our experiments, no increased protein nitration by endogenously formed peroxynitrite was found in cells grown on low glucose. This results is apparently conditioned by low yield of nitration under physiological and cell culture conditions. It has been reported that even under inflammatory conditions, one to five 3-nitrotyrosine residues per 10,000 tyrosine residues are detected [40,41], due mainly to repair mechanisms of the tyrosyl radical such as reduction by glutathione and to metabolism of 3-nitrotyrosine.

The small fraction of nitrated protein under physiological conditions cast doubt on its possible biological relevance. It is noteworthy to indicate, however, that a relatively limited number of proteins are preferential targets of nitration, and within these proteins, only one or a few specific tyrosines can be nitrated.

Addition of a $-NO_2$ group to tyrosine lowers the pK_a of its phenolic hydroxyl group by 2–3 units and adds a bulky substituent; if placed on relevant tyrosines, nitration can alter protein function and conformation, impose steric restrictions, and also inhibit tyrosine phosphorylation. However, to have biological significance, a loss-of-function modification requires a large fraction of protein to become nitrated at specific critical tyrosines and result in 3-nitrotyrosine to tyrosine ratios in the range of 0.1–1.0; documentation of these large ratios for a given protein *in vivo* is scanty, and it is doubtful that many proteins will undergo such an extent of nitration.

Our study demonstrates effective removal of nitrotyrosine from cellular proteins nitrated by exogenous peroxynitrite (Fig. 5). In the work of Aghdam et al. [42] it was shown that activity of proteasome is directly connected with glucose levels in intracellular environment; proteasome activity is elevated at higher glucose concentration. Our research shows that glucose concentration in culture media influences the rate of removal of modified proteins. Higher concentration of glucose promotes faster degradation of nitrated proteins. AgNPs increase the rate of degradation at lower glucose concentration due to oxidative stress [43] activating NRF2 signaling pathway, which in turn elevates proteasome activity [44].

The presence of a nitoryrosine denitrase activity, independent of digestion of nitrated proteins, was first reported by Kamisaki et al. [45]. According to the present knowledge, denitrase activity can be described as an oxygen-regulated and endotoxin-induced tissue-specific process, detectable in brain, spleen, and lung of LPS-treated rats, in untreated rat heart and brain as well as in LPS-treated RAW 264.7 cells [46]. The present strongly suggest that the denitrase activity is present also in HepG2 cells and is higher in cells grown on high glucose. However, since we did not use protease inhibitors, it cannot be excluded that the removal of nitrotyrosine residues is partly dependent on proteolysis of nitrated proteins.

The in vitro test (Fig. 6) demonstrated that the presence of silver nanoparticles attenuates nitrotyrosine formation, apparently due to peroxynitrite decomposition. However, in the cellular system the presence of AgNPs did not protect cellular proteins against nitration, in cells grown on low or high glucose. Apparently, more efficient peroxynitrite-decomposing mechanisms present in the cells make the role of AgNPs marginal in cellular systems, in contrast to the simple system of peroxynitrite + tyrosine. The increased de-nitration of proteins in cells grown on low glucose incubated with AgNPs is noteworthy. There are no data on denitrase activity of AgNPS and such a mechanism does not seem probable. Rather, AgNPs can be expected to induce synthesis of defensive proteins which may include those showing denitrase activity or proteolytic activity.

Lipid peroxidation can be described as an oxidation of lipids containing double carbon-carbon bonds leading to formation of peroxides. Most susceptible to peroxidation are polyunsaturated fatty acids (PUFAs), but other lipids like glycolipids, phospholipids (PLs) and cholesterol can undergo the process as well. Lipids can be oxidized in enzymatic processes, involving lipoxygenases, cyclooxygenases and cytochrome P450 and non-enzymatically in a chain process initiated by free radicals.

Lipid peroxidation was initially studied as a deleterious outcome of oxidative stress, however the regulatory aspect of the process was also noticed [47]. Lipid peroxidation is a physiological process proceeding under physiological conditions but is enhanced in oxidative stress. Under conditions of mild oxidative stress, lipid peroxidation products upregulate antioxidant protection system which in turn limits the harmful effects of oxidative stress. However intensified lipid peroxidation can lead to cell death by apoptosis, necrosis or autophagy [48–50]. Most extensively studied secondary products of lipid peroxidation are malondialdehyde (MDA), propanal, hexanal and 4-hydroxynonenal (4-HNE). MDA and 4-HNE were described respectively as most mutagenic and most toxic products of lipid peroxidation [51]. 4-HNE is considered as one of the major lipid peroxidation products and mediating secondary effects of oxidative stress [52]. High toxicity of 4-HNE is tied with its high reactivity against thiol and amino groups [53]. It was found that 4-HNE upregulates many transcription factors e.g. nuclear factor erythroid 2-related factor 2 (Nrf2) [54–56], activating protein-1 (AP-1) [57], NF-κB [58] and peroxisome-proliferator-activated receptors (PPAR) [59,60], mitogen-activated protein kinases (MAPK) and other stress response pathways [61]. Non-physiological levels of 4-HNE can promote development and progression of pathological states such as neurodegenerative diseases [62], diabetes mellitus [63], and carcinogenesis [64,65]. In our previous work [17] we have shown that change in environment glucose level promotes cell adaptation to oxidative stress. 4-HNE can be one of the key signaling molecule behind this adaptation process, the more that nanoparticles have been

demonstrated to promote lipid peroxidation [66].

In summary, AgNPs do not induce nitrative stress in HepG2 cells but even inhibit nitration in a cell-free system, most probably by reacting with peroxynitrite (although this effect seems to be of no importance in cellular systems) and accelerate the de-nitration of tyrosine residues. Apparently, the action of AgNPs in HepG2 cells is not direct but mediated by induction of biosynthesis of defensive proteins, especially in cells grown in low glucose. This question requires further studies.

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Exposure of human neurons to silver nanoparticles induces similar pattern of ABC transporters gene expression as differentiation: Study on proliferating and post-mitotic LUHMES cells

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ABSTRACT

The Lund human mesencephalic (LUHMES) cell line originated from mesencephalon of 8-week human foetus is a renowned in vitro model of human dopaminergic neurons. After differentiation the cells exhibit dopaminergic and neuronal characteristics of biochemically and morphologically mature dopamine-like neurons. In this study we analysed expression of 42 genes from ABC transporter superfamily in both proliferating cells and differentiated neurons after treatment with silver nanoparticles. ABC transporter superfamily is especially known due to the involvement in multidrug resistance phenomenon, but also involvement in transport through blood-brain barrier. Our results indicate that in neurons silver nanoparticles mainly attenuate transporters responsible for maintaining asymmetry of cellular membrane and homeostasis of lipids and cholesterol. Our results revealed also that proliferating foetal brain cells are by far more susceptible to silver nanoparticles than differentiated neurons.

1. Background

ABC transporters are highly conserved, ubiquitous proteins, most of which are transmembrane molecules. The majority of proteins in this family consist of at least one nucleotide binding domain (NBD) and one transmembrane domain (TMD). Full transporters consist of two of each, half transporters have one NBD and one TMD and they must homo- or heterodimerize to be biologically functional (Dean et al., 2001; Higgins, 1992). They use the energy of ATP hydrolysis to translocate a broad spectrum of compounds across the cellular and intracellular membranes, including but not limited to inorganic anions, metal ions, peptides, amino acids and sugars (Slot et al., 2011). However, the function of these proteins is not limited to the transport through membranes, e.g. proteins of ABCA subfamily are involved in apoptosis signalling pathway by presenting specific lipids on the outer plasma membrane (Aye et al., 2009), while members of subfamilies lacking TMD (ABCE and ABCF) lack of transport function and play important roles in translation (Kerr, 2004; Tyzack et al., 2000). In mammals, ABC transporters are expressed predominantly in the liver, intestine, blood-brain barrier, blood-testis barrier, placenta and kidney but their expression was also confirmed in neurons.

Presence of particular ABC proteins is closely tied with function of given cells. Neurons and oligodendrocytes express transporters involved in translocation of lipids (Schmitz and Kaminski, 2002; Tanaka et al., 2003), glutamate (Domercq et al., 1999; Kanai and Hediger, 2003) and amino acids (Braissant et al., 2001; Mackenzie and Erickson, 2004). In addition, expression of ABCC1 (MRP1) transporter was found on membranes of microglia and astrocytes (Dallas et al., 2003; Decleves et al., 2000), the cells associated with loss of neurons during the development of neurodegenerative diseases (Nagele et al., 2004; Teismann and Schulz, 2004). Both are responsible for apoptosis of neurons in response to oxidative stress and inflammation, and are a therapeutic target in treatment of neurodegenerative diseases (Kitamura and Nomura, 2003; Liu and Hong, 2003; Monsonego and Weiner, 2003). Sultana and Butterfield found elevated expression of ABCC1 (MRP1) proteins in frozen hippocampal samples from patients suffering from Alzheimer's disease as compared with aged male subjects without neurodegenerative changes (Sultana and Butterfield, 2004). Recently, also the activity of other ABC transporters was tied to the development and progression of Parkinson and Alzheimer diseases

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(Furuno et al., 2002; Lam et al., 2001; Vogelgesang et al., 2002).

Though, many aspects of the aetiology of neurodegenerative disorders still need clarification, ethical issues and implementation of 3R rule in animal care that limits the use of animals and primary cells in biological research, make such investigation difficult. Thus, there is an increasing interest in the *in vitro* cellular models, mimicking CNS functions. So far, cancer neuronal cells, such as human neuroblastoma cell lines (SH-SY5Y or BE2-M17) or rat pheochromocytoma PC12 cells, are the most commonly studied *in vitro* models of neuron-like cells. However, due to their cancer origin, these cells are to a large extent different in the phenotype from mature post-mitotic neurons and need to be transcriptionally reprogrammed by addition of diverse factors into the culture media, such as NGF or retinoic acid. As an alternative, the Lund human mesencephalic (LUHMES) cells were generated to be used for neuronal transplantation in Parkinson's disease (Lotharius et al., 2002; Lotharius et al., 2005). As the LUHMES cells properties closely resembles those of dopaminergic neurons *in vivo*, this model can serve for basic research on processes associated with neurodegenerative diseases, but also as a screening system within neurotoxicological testing programs. However, whereas, the general neuronal characteristics and differentiation status of LUHMES cells at different culture conditions was already elaborated (Paul et al., 2007; Schildknecht et al., 2009), their comprehensive characterization still await for completion. Increased body of evidence points to the detrimental role of nanomaterials in the functionality of CNS (Czajka et al., 2015) and their possible role in neurodegenerative diseases aetiology (Mushtaq et al., 2015). Nanomaterials in general and silver nanoparticles in particular are widely used in industry and exposure of humans and other organisms towards them grows in time. Nevertheless, the questions rose in last decade on impact of nanomaterials on human health are still unanswered (Manke et al., 2013). It has been shown that nanoparticles can penetrate blood-brain barrier (Oberdorster et al., 2004) and accumulate in nervous and liver tissues (Dziendzikowska et al., 2012; Geiser and Kreyling, 2010). Inside the cells nanoparticles influence variety of cellular structures, *inter alia*, the mitochondria. One of the major effects of damaging mitochondria is the increase in production of reactive oxygen species that can further damage cell structures and cause the cascade of activation of intracellular signalling, including NF-κB, Ap-1, MAPK, PTP and ERK pathways (Bartlomiejczyk et al., 2013; Manke et al., 2013). Activation of signalling pathways can lead to changes in expression of ABC transporters, in particular those involved in response to oxidative stress. Moreover, reorganization of cellular membrane in effect of endocytosis of nanoparticles can cause changes in activity of ABC transporters tied with cellular membrane.

A ubiquitous presence of ABC transporters across variety of cells in CNS infers their important role in CNS functionality, but also rises question of its role in the detrimental effects of nanomaterials observed in the CNS. Nanomaterials are known to penetrate the brain-blood barrier and placental barrier, but their interaction with ABC transporters in differentiated and developing brain is still obscure. Thus, the aim of this study was to investigate the effect of silver nanoparticles on expression of several ABC-transporter proteins in differentiated and proliferating LUHMES cells and to compare the changes induced by AgNPs with those observed during differentiation. However, since differentiation of the LUHMES cells is induced by addition of tetracycline, an antibiotic that blocks protein synthesis, in this study we focused only on changes in mRNA level.

2. Materials and methods

2.1. Cell culture

LUHMES cells were grown in monolayer in NunclonD™ cell culture flasks coated with poly-L-ornithine (50 μg/cm^3) and human plasma fibronectin (1 μg/cm^3) (Sigma-Aldrich) as described in our previous study (Stepkowski et al., 2016). Differentiation was conducted according to the previously established two-step protocol (Scholz et al., 2011). Detailed description of LUHMES cells culture and differentiation can be found in supplementary materials.

2.2. AgNP preparation, characterization and treatment

The stock suspension (2 mg/cm^3) of the AgNPs of a 20 nm nominal diameter (PlasmaChem, Germany) was prepared as previously described (Kruszewski et al., 2013). In brief, bulk AgNPs were triturated in agate mortar, then 2 mg of NPs was suspended in 800 mm^3 of water. Suspension was sonicated (4.2 kJ/cm^3) with OmniRuptor 4000 and then 100 mm^3 of 15% albumin which was followed by addition of 100 mm^3 of tenfold concentrated PBS (1.37 M NaCl, 27 mM KCl, 80 mM Na_2HPO_4, 20 mM KH_2PO_4). A detailed characterization of this batch of AgNPs, including different preparation protocols, dispersion, aggregation and stability in different culture media, as well as silver ion release, was previously described (Kruszewski et al., 2013). A pre- and post-mitotic (differentiated) cells grown in 25 cm^2 flasks were treated with vehicle (BSA + PBS) or AgNPs stock solution dispersed in the 4 cm^3 of culture medium to the final concentration of 2 μg [AgNPs]/cm^3 [medium] for 6 h.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from cell pellets quick-frozen in liquid nitrogen, with the use of ReliaPrep™ RNA Cell and Tissue Miniprep System according to the manufacturer protocol (Promega). RNA integration and concentration was assessed by gel electrophoresis and in Quantus Fluorometer (Promega), respectively. cDNA was synthesized with GoScript cDNA synthesis kit with the use of a mixture of OligodT and random primers according to the manufacturer protocol (Promega). DNA polymerase was heat inactivated for 15 min at 70 °C.

2.4. Gene expression studies

Gene expression of ABC family of proteins was assessed with commercially available panel of prevalidated qPCR assays for gene expression profiling of human genes − ABC Transporter Panel, 96 (Roche, Cat. No. 05 339 324 001). Experimental procedures were conducted accordingly to manufacturers guidelines.

Significance was tested via Pair Wise Fixed Reallocation Randomization Test using REST 2009 software (Pfaffl et al., 2002). Treated samples and control samples were seeded, collected and processed in three independent experiments.

3. Results

Expression of 32 genes coding ABC transporter proteins belonging to the ABCA, ABCB, ABCC, ABCD, ABCE and ABCF families was studied in relation to cells differentiation status and silver nanoparticle treatment. A detailed list of genes studied in this research including changes in their expression is presented in Supplementary materials (Supplementary Table S1). Here we discuss only genes whose transcription changed significantly.

Our results indicate that differentiation process induces a dramatic change in expression of mRNA of all tested genes belonging to ABCA and several genes belonging to ABCC: *ABCC5*, *ABCC8*, *ABCC10*, *ABCC11*. A marked change in expression was also observed in case of *ABCB9*, *ABCD1*, *ABCD4*, *ABCF3* genes. Once cells differentiated, they lost expression of *ABCB4*, but started to express *ABCA6* and *ABCG4*, *ABCG5* and *ABCG6* genes (Fig. 1). Since tetracycline was used to differentiate the LUHMES cells, the question arose whether this treatment would not stimulate expression of mammalian multidrug resistance proteins, namely ABCB1 (MDR1), ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3), ABCC4 (MRP4), ABCC5 (MRP5) and ABCG2 (BCRP) (Schinkel and Jonker, 2003). Comparison of ABC transporters mRNA

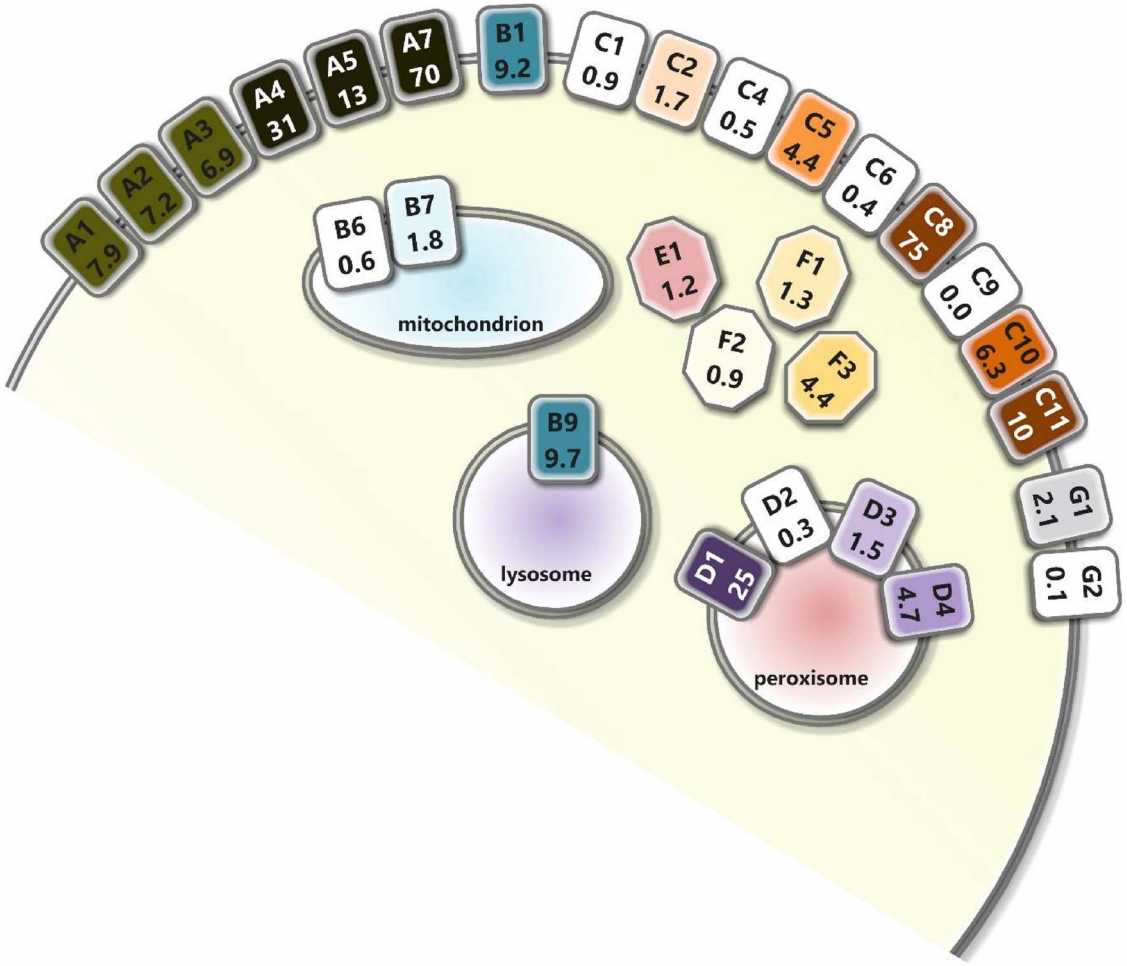

Fig. 1. Shift of ABC transporters expression profile in LUHMES cells after differentiation process. Numbers in rectangles represent fold-change in expression of given protein on mRNA level. Location of the rectangles corresponds to the location of mature proteins. ABC family transporters are grouped colour-wise, while intensity corresponds to magnitude of fold-change in expression. Statistically significant changes were found for ABCA4, ABCA5, ABCA7, ABCB9, ABC11 and ABCG2.

levels in proliferating and differentiated (tetracycline treated) cells revealed that although expression of ABCB1, ABCC5 was stimulated after addition of tetracycline, these changes were not significant statistically. The only statistically significant change in expression of multidrug resistance proteins observed by us after tetracycline addition was down-regulation of ABCG2 protein mRNA.

A non-differentiated cells are far more susceptible to silver nanoparticles treatment than the post-mitotic ones in terms of change of ABC transporters genes expression. The major changes were observed in mRNA levels for ABC proteins crucial in cholesterol transport, stabilization of membrane asymmetry and those regulated by oxidative stress, including (Fig. 2). Interestingly, treatment with AgNPs induced changes in ABC transporters genes expression very similar to those observed during differentiation process, however to the lesser extent (Figs. 1 and 2). Since tetracycline was used to differentiate the LUHMES cells, the another question arose whether this treatment would not induce a oxidative stress similar to this induced by silver nanoparticles, thus observed similarities result from similar mode of action of both compounds. However, literature survey revealed that this was not the case, as tetracycline was shown to scavenge free radicals (especially hydroxyl radical) rather than generate oxidative stress, in cells and *in vitro* (Miyachi et al., 1986).

Below changes in gene expression are discussed in detail in regards to their affinity and putative role.

4. Discussion

There is increasing body of evidence that among many other effects on living organisms nanomaterials may influence protein expression, both on transcriptional (mRNA) and translational (protein) levels (Matysiak et al., 2016). AgNPs exposure induced expression of oxidative stress response genes (superoxide dismutase 2, glutathione reductase 1, etc.) in mouse brain (Rahman et al., 2009). AgNPs were also reported to induce changes in mRNA levels of inflammatory and neurodegenerative responses in mouse brain neural cells (Huang et al., 2015). Here we describe the effects of silver nanoparticles on expression of ABC transporters genes in human neurons in culture. The major changes were observed in expression of ABCA and ABCC transporter families gene, thus these genes will be described in details.

4.1. Transporters of ABCA subfamily

ABCA1 is a transporter involved in formation of high density lipoproteins (HDLs) and plays a crucial role in sustaining proper cholesterol metabolism (Oram and Lawn, 2001). During differentiation, astrocytes and neurons produce cholesterol, which is essential in the formation of myelin. Along with the progress of differentiation biosynthesis of cholesterol slows down, nevertheless, even in the mature neurons cholesterol is still indispensable in terms of maintaining vital functions and normal fluidity of cell membrane (Vitali et al., 2016). It has been estimated that approximately 25% of the cholesterol is held in the brain and nervous system, indicating a remarkable role of this molecule in

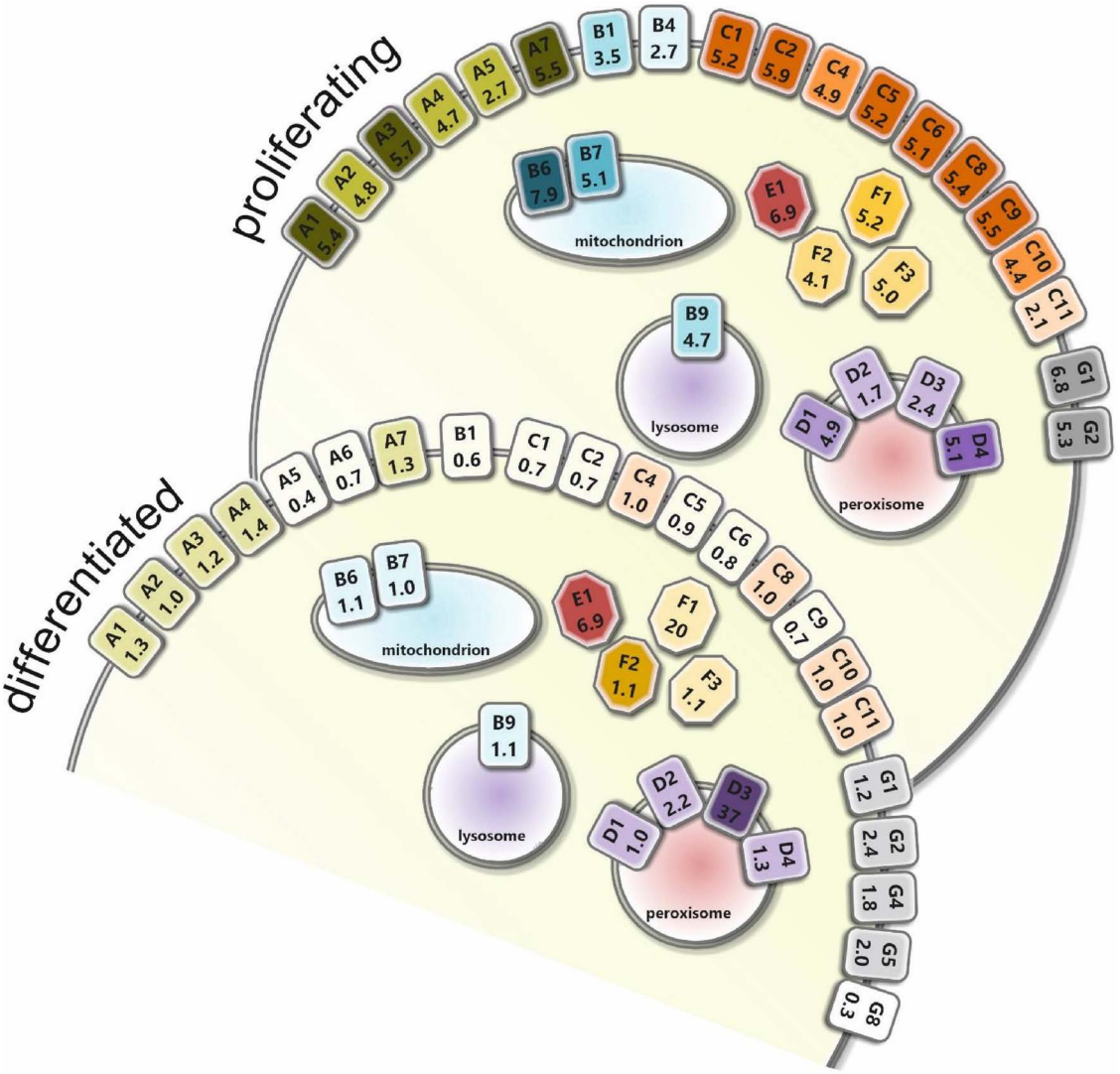

Fig. 2. Change in mRNA levels for ABC transporters in LUHMES cells subjected to 20 nm AgNP (6 h, 2 μg/cm^3) treatment before (A) or after (B) differentiation. Numbers represent fold-change in expression of given protein on mRNA level after AgNP treatment. Location of the rectangles corresponds to the location of mature proteins. ABC transporters belonging to the same protein family are grouped colour-wise, while colour intensity corresponds to the magnitude of change in expression. Statistically significant changes were found in expression of ABCA1, ABCA4, ABCA7, ABCB4, ABCB6, ABCB9, ABCC2, ABCC6, ABCC9 and TNFAIP2 for proliferating cells and in expression of ABCD3 for differentiated cells.

maintaining homeostasis of nervous system cells. The key role of ABCA1 in lipid metabolism was confirmed by studies on cell lines. In vitro studies implicated the role ABCA1 in the efflux of cholesterol and phospholipids from cells to lipid-poor apolipoprotein A1 (apoA-1) to generate nascent HDL, a pathway termed reverse cholesterol transport. In CNS ABCA1 regulates transport of cholesterol and phospholipids thereby facilitating lipidation of ApoE, which is a major apoprotein in the brain (Hirsch-Reinshagen et al., 2004; Panzenboeck et al., 2002). In consequence, ABCA1-deficiency results in lowered lipidation of ApoE and in overall decrease of ApoE levels in the brain (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). RT-PCR based studies revealed that neurons express predominantly *ABCA1* and *ABCA3* mRNA; astrocytes express *ABCA1*, *ABCA2* and *ABCA3* mRNA; microglia express *ABCA1* mRNA and oligodendrocytes express *ABCA2* and *ABCA3* mRNA (Kim et al., 2006). In this study expression of *ABCA1* increases during differentiation. Interestingly also treatment of proliferating cells with silver nanoparticles also increases the level of *ABCA1* mRNA (Fig. 2). We associate this phenomenon with disturbed membrane architecture caused by endocytosis of nanoparticles (Fig. 3). A consequence of increased endocytosis is the return of the membrane of the lysosomes to plasma membrane and ER and enhanced ABCA1 turnover (Cooper, 2000). Increased level of ABCA1 mRNA might be a compensation mechanism to keep the protein on the necessary level.

Moreover, endosomal ABCA1 protein may facilitate ejection of cholesterol from endosomes. A liberated cholesterol particles are likely oxidized by AgNPs derived ROS to oxysterols that in turn stimulate ABCA1 expression through a Liver X receptor (LXR) transcription factor (Fig. 3). LXRs are directly involved in regulation of transcription of ApoE and ABC transporters responsible for normal lipid composition of ApoE, such as ABCA1 and ABCG1 (Wang et al., 2014). Normal levels of cholesterol, both in cytosol and cell membrane guarantees the proper functioning of nerve cells, whereas any disturbances in distribution of lipids, are linked with many diseases, e.g. Alzheimer disease and Huntington disease (Markianos et al., 2008).

ABCA4 transporter plays important role in proper functioning of retinal photoreceptors. In retinal photoreceptor cells it actively flips *N*-retinylidene-phosphatidylethanolamine from the lumen to the cytoplasmic leaflet of disc membranes, and transport of phosphatidylethanolamine in the same direction (Quazi et al., 2012). However, ABCA4 activity is also associated with lowering of oxidative stress. In tissues of ABCA4(-/-) mice mRNA levels for antioxidative enzymes were increased, whereas the levels of oxidized proteins and lipids were increased when compared to WT mice (Radu et al., 2011). Promoter region of *ABCA4* gene consists of binding sites for oxidative stress

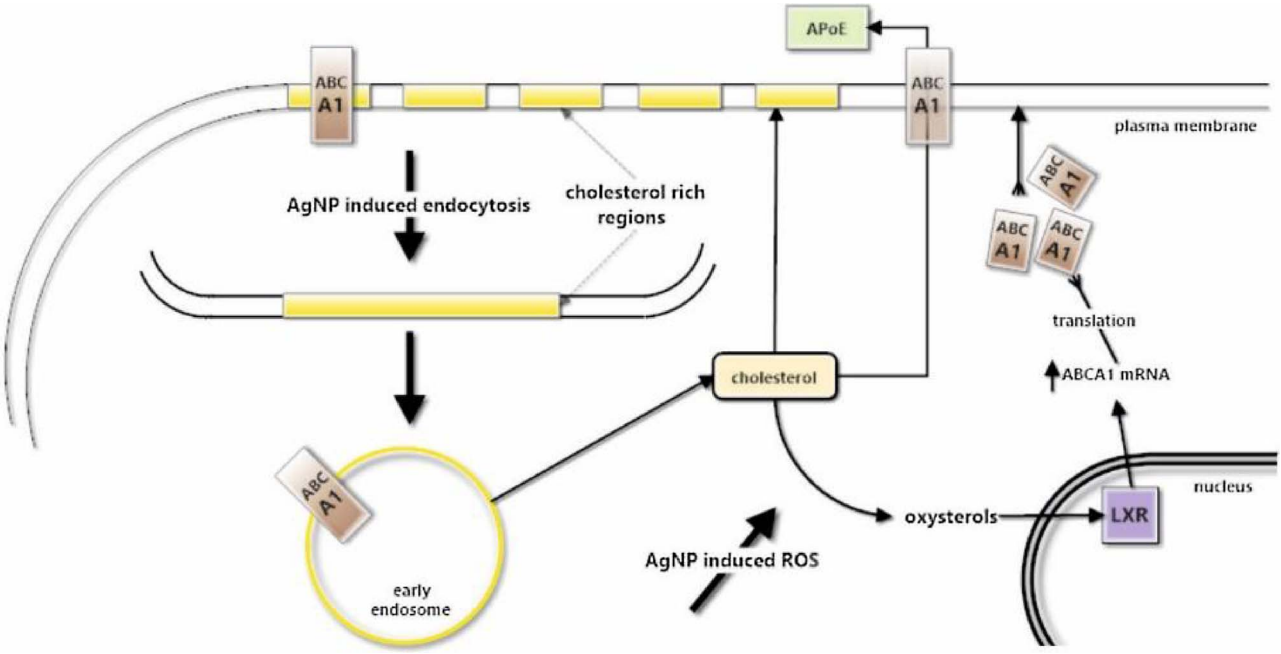

Fig. 3. Migration and function of ABCA1 in cells treated with silver nanoparticles.

inducible transcription factors, such as STAT1, NF-κB. Since AgNPs were shown to strongly induce both NF-κB and JAK-STAT signalling pathways (Xu et al., 2012) (Stepkowski et al., 2014), and the pathway is also activated during embryonal stem cells neuronal differentiation (Wei et al., 2014), increased expression of *ABCA4* mRNA during differentiation and after AgNPs treatment could indicate the prior activation of NF-κB or JAK-STAT1 pathways, which is essential in regulating the stress response.

ABCA7 is a protein widely expressed in the brain, lung, myelolymphatic tissues, kidneys, macrophages and platelets and it shows 54% similarity with the ABCA1 (Kim et al., 2006). It is postulated, that both proteins might share the substrate specificity and function (Abe-Dohmae et al., 2006). However, some studies indicate that ABCA7 is responsible for efflux of phosphatidylcholine and sphingomyelin rather than cholesterol (Wang et al., 2003). ABCA7 is intensively studied protein due to its connection with Alzheimer's disease, as an absence of ABCA7 results in reduced ability of macrophages to phagocytose Aβ and accumulation of insoluble Aβ in brain (Li et al., 2015). ABCA7 promoter region contains binding sites for E47 and TAL-1 transcription factors. Phosphorylation of E47 is regulated by p38 MAP-Kinases pathway (Cuenda and Rousseau, 2007) that in turn is activated by oxidative stress generated by AgNPs. Activation of p38 MAPK was proposed as a mechanism of toxicity of silver nanoparticles in Jurkat T cells (Eom and Choi, 2010). On the other hand, activity of the transcription factor TAL-1 is linked with activation of the EGFR pathway (Zhou et al., 2014) that is also associated with oxidative stress (Wang et al., 2000). Given that it is possible that silver nanoparticle generated oxidative stress activate the above mentioned signalling pathways, and in consequence regulate the mRNA levels of *ABCA7* gene.

The expression of ABCA subfamily genes was the most affected by silver nanoparticles among studied transporters. This demonstrates the intense disorder of cholesterol metabolism caused by silver nanoparticles and significant interference with dynamics of cell membrane. Nanoparticles have the ability to cross the placenta (Chu et al., 2016; Kulvietis et al., 2016) and it was found that exposure of laboratory animals to nanoparticles resulted in changes in foetus expression profile of genes associated with brain development, cell death, oxidative stress and inflammation (Shimizu et al., 2009). Observations collected during experiments on laboratory animals in conjunction with ours results indicate that nanoparticles can significantly influence differentiating nerve cells in human foetuses.

4.2. Transporters of ABCB subfamily

ABCB family proteins form elements of blood – brain barrier in endothelial cells. Differentiation of LUHMES cells induced a marked increase in expression of *ABCB1* and *ABCB9* mRNA. Associated with outer membrane ABCB1 protein is usually considered in the context of multi-drug resistance in CNS and in tumour cells. In contrary, ABCB9 transporter seems to be bound to lysosomes, although its exact localization is still unclear (Demirel et al., 2007). ABCB1 protein is expressed on the surface of human neural stem/progenitor cells, but its expression decreases during cell differentiation to astrocytes (Islam et al., 2005) whereas ABCB9 expression has been found to increase during the differentiation of monocytes into dendritic cells (Demirel et al., 2007). Here we showed that during differentiation to dopaminergic neuron expression of *ABCB1* and *ABCB9* genes is sustained. Expression of ABCB1 is also regulated by oxidative stress. Hydrogen peroxide activates signalling through ERK1/2, PKC, Akt, c-Jun and NFκB, which turns on ABCB1 transcription (Nwaozuzu et al., 2003). Increased production of oxygen free radicals is induced by toxins and nanomaterials and is associated with many CNS diseases. In capillaries isolated from rat brains fuel nanoparticles induced increase in expression and activity of ABCB1 and increase in NADPH activity (Hartz et al., 2008). Oxidative stress is closely tied to CNS diseases by ability to activate TNF-alpha, TNF-R1, JNK and c-Jun dependent signalling pathways. Thus, it is likely that silver nanoparticles induced oxidative stress also stimulates transcription of *ABCB1* and *ABCB9* mRNA.

Treatment of non-differentiated cells with AgNPs also induced expression of *ABCB4*, *ABCB6*, *ABCB7* genes. Although the presence of mRNA for *ABCB4*, *ABCB6*, *ABCB7*, *ABCB8* and *ABCB10* has been demonstrated in brain homogenates (Warren et al., 2009) and ABCB4 and ABCB11 (bile salt export pump, BSEP) mRNA has been detected in human and rat choroid plexus (Choudhuri et al., 2003; Niehof and Borlak, 2009), there are no evidence however for expression of genes ABCB2-11 on protein level in CNS. There is also little information available on expression of these genes in particular kind of brain cells, including neurons.

ABCB4 is involved in metabolic pathway of bile secretion and regulation of lipid metabolism by peroxisome proliferator-activated

receptor alpha. The main physiological role on cellular level of ABCB4 is the maintenance of correct lipid composition of both non-raft membranes and membrane rafts (Morita et al., 2013). ABCB6 transporter is commonly found in mammalian cells. The level of mRNA for this protein increases during hepatocarcinogenesis (Furuya et al., 1997; Hirsch-Ernst et al., 1998). ABCB6 localizes in mitochondria and plays a key role in heme biosynthesis (Crawford et al., 1997). Regulation of ABCB6 expression is complex issue. One of the major signalling pathways influencing ABCB6 expression is NRF1 pathway, which is closely tied with regulation of genes expression associated with biogenesis and correct functioning of mitochondria (Scarpulla, 2002). This suggest that ABCB6 is expressed strongly in rapidly proliferating cells with need for rapid synthesis of mitochondrial proteins.

Here we showed that human proliferating neural cells are capable for expression of *ABCB4*, *ABCB6*, ABCB7, *ABCB9* genes. Weather this mRNA is translated into proteins or degraded needs further investigation. Here we showed that human proliferating neural cells are capable for expression of *ABCB4*, *ABCB6*, ABCB7, *ABCB9* genes. Weather this mRNA is translated into proteins or degraded needs further investigation. Moreover, we found that mRNA levels for several members of ABCB subfamily increased after AgNP treatment (Fig. 2), thus implying a possible role of oxidative stress in regulation of its expression. Indeed, promoter region of ABCB6 gene contains a putative binding sites for AP-1 and NFkB transcription factors (see GeneCards Database ID:GC02M220075). Moreover, CHiP-sequencing revealed that in isothiocyanate sulforaphane treated cells ABCB6 gene is also bound by NRF2 transcription factor (Campbell et al., 2013).

4.3. Transporters of ABCC subfamily

The ABCC1, ABCC4 and ABCC5 proteins were clearly localized in brain capillary endothelial cells (Nies et al., 2004), the ABCC3, ABCC4 and ABCC5 proteins were also detected in astrocytes (Hirrlinger et al., 2002; Hirrlinger et al., 2005; Nies et al., 2004), whereas ABCC5 protein was present in pyramidal neurons (Nies et al., 2004), with ABCC1 and ABCC5 being most predominant in brain cells among ABCC family. ABCC1 was the first C protein discovered in CNS (Regina et al., 1998), and was extensively studied over the years mainly due to its role in multidrug resistance phenomena. ABCC1 regulation is tied to oxidative stress responsive signaling pathway, such as MAPK, JNK/STAT and NFκB (Ronaldson et al., 2016). Indeed, in our study we observed significant increase in the level of ABCC1 mRNA in proliferating cells treated with silver nanoparticle. Interestingly, upregulation of *ABCC1* gene expression was negligible either in differentiated cells treated with AgNPs or during differentiation. We have also observed strong increase in the level of *ABCC2*, *ABCC6* and *ABCC9* mRNA. ABCC2 has been detected in the blood-brain barrier. It was reported that exposure of capillaries isolated from rat brain to the pregnenolone-16α-carbonitrile and dexamethasone causes increased activity and expression of ABCC2 (Bauer et al., 2008). In cultured endothelial cells derived from rat brain capillary dexamethasone caused upregulation of ABCC2 mRNA and protein in glucocorticoid receptor activation independent manner (Narang et al., 2008). ABCC9 transcript SUR2 is expressed in variety of tissues, including brain. On cellular level it regulates function of K^+ channels and it is linked, on organismal level, to hippocampal sclerosis of aging (Nelson et al., 2015). Potassium channels can be regulated in ROS-dependant manner(Crawford et al., 2003), observed upregulation after AgNPs treatment suggest that oxidative stress dependant pathway might be involved in tuning of expression of these proteins.

4.4. Transporters of ABCD subfamily

Member proteins of ABCD transporter subfamily are located in the peroxisomal membrane, where they transport fatty acids into peroxisomes for metabolic breakdown (Theodoulou et al., 2006). The presence of ABCD1-4 mRNAs was confirmed in mouse brain capillary endothelial cells, but only ABCD1 was detected on the protein level (Berger et al., 1999). ABCD1 was also found in astrocytes, microglia and oligodendrocytes from mouse brain and post-mortem human brain sections (Fouquet et al., 1997). ABCD3 is one of the peroxisomal membrane proteins, detected in abundance in hepatocytes and was reported to transport various fatty acids. Overexpression of the ABCD2 or ABCD3 gene can reverse the biochemical phenotype of X-ALD (reduced beta-oxidation of very-long-chain fatty acids) (Fourcade et al., 2001). ABCD3 along with ABCD2 was show to being upregulated to compensate the lack of ABCD1 after treatment of astrocytes and oligodendrocytes with histone deacetylase inhibitor.

There is no literature on the mechanisms of regulation of expression of ABCD subfamily genes. Again our results imply oxidative stress involvement in this regulation.

4.5. Transporters of ABCG subfamily

Similarly, little is known about regulation of expression and the role in CNS of proteins belonging to ABCG family. Wang et al. (Wang et al., 2008) proposed that proteins of this family function as a of cholesterol, sterols an sterol complexes transporters. In addition, ABCG2 is an element of multidrug resistance maintenance of integrity of the blood-brain barrier. However, after addition of tetracycline (to differentiate the LUHMES cells) we observed downregulation of *ABCG2* gene expression, thus it seems that in line with neural stem cells (Islam et al., 2005), in neurons ABCG2 is likely responsible for detoxification of xenobiotics. A putative role of ABCG in our experimental setup is regulation of cholesterol homeostasis. It is supported by the fact that we detect mRNA for those proteins only in differentiated cells, where de novo synthesis of sterols is hindered in comparison to undifferentiated cells.

5. Summary

In this work we compared ABC transporters mRNA levels in proliferating and non-proliferating human neuronal LUHMES cells native and treated with silver nanoparticles. Our results indicate that differentiation induced marked changes in expression of several genes coding proteins belonging to ABC transporter protein family. Interestingly, in rapidly proliferating cells silver nanoparticles treatment induced similar changes in ABC transporters gene expression as differentiation. On the contrary, changes in ABC transporters mRNA level after silver nanoparticles treatment in post-mitotic cells were much less pronounced that may indicate similarities in cellular signalling pathways stimulated by differentiation and silver nanoparticle treatment. Although we were unable to confirm our results at protein level due to the unavailability of antibodies against several crucial ABC proteins, our results point to the possible new impact of silver nanoparticles on human health, especially on developing brain. Nevertheless, it must be noted that posttranscriptional and posttranslational regulatory mechanisms may affect the final expression of ABC transporters at the protein level, and may modify the effects of silver nanoparticles treatment observed at the transcriptional level.

Declarations of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mad.2018.02.004.

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Banacha 12/16, 90-237 Łódź, Polska

Łódź 16/05/2017

OŚWIADCZENIE

Oświadczam, że w pracy:

Glucose availability determines silver nanoparticles toxicity in HepG2. **Zuberek M**, Wojciechowska D, Krzyzanowski D, Meczynska-Wielgosz S, Kruszewski M, Grzelak A,

Mój udział polegał na przeprowadzeniu testów przeżywalności, określeniu poziomu mitochondrialnego nadtlenku wodoru, oznaczeniu aktywności enzymów antyoksydacyjnych, opracowaniu koncepcji badań, statystycznej analizie danych oraz na przygotowaniu manuskryptu publikacji. Mój wkład w powyższą pracę określam na 55%.

Podpis:

Mgr Dominika Wojciechowska

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Mój udział polegał na wykonaniu wstępnych oznaczeń przeżywalności i oznaczeń mitochondrialnego nadtlenku wodoru. Mój wkład w publikację oceniam na 1 %

Podpis:

Wojciechowska

Mgr Damian Krzyżanowski

Wydział Biologii i Ochrony Środowiska

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Podpis:

Warszawa 16/05/2017

Dr Sylwia Męczyńska-Wielgosz
Instytut Chemii i Technik Jądrowych
Dorodna 16, 03-195 Warszawa, Polska

OŚWIADCZENIE

Oświadczam, że w pracach:

Glucose availability determines silver nanoparticles toxicity in HepG2. Zuberek M, Wojciechowska D, Krzyzanowski D, **Meczynska-Wielgosz S**, Kruszewski M, Grzelak A,

mój udział polegał na charakterystyce nanocząstek srebra pod względem fizycznym. Mój udział oceniam na 2%.

Podpis: S. Męczyńska-Wielgosz

Warszawa 16/05/2017

Prof. dr hab. Marcin Kruszewski
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OŚWIADCZENIE

Oświadczam, że w pracy:

Glucose availability determines silver nanoparticles toxicity in HepG2. Zuberek M, Wojciechowska D, Krzyzanowski D, Meczynska-Wielgosz S, **Kruszewski M**, Grzelak A,

mój udział polegał na uczestniczeniu w opracowaniu koncepcji badań, wprowadzaniu poprawek do manuskryptu i zatwierdzeniu ostatecznej wersji do złożenia. Swój udział w tej pracy oceniam na 1%.

Podpis:

Dr Agnieszka Grzelak

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Mój udział polegał na opracowaniu koncepcji badań, wprowadzeniu poprawek do manuskryptu i zatwierdzeniu ostatecznej wersji do złożenia. Swój udział oceniam na 40%.

Podpis:

Mgr Mariusz Żuberek

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Łódź 16/05/2017

OŚWIADCZENIE

Oświadczam, że w pracy:

Silver baboparticles can attenuate nitrative stress. **Zuberek M**, Paciroek P., Bartosz G., Grzelak A.

Mój udział polegał na przeprowadzeniu testów przeżywalności, określeniu ilości mRNA dla wybranych genów, pomiarach reaktywnych form tlenu i azotu, oznaczeniu stężenia nitrotyrozyny, opracowaniu koncepcji badań, statystycznej analizie danych oraz na przygotowaniu manuskryptu publikacji. Mój wkład w powyższą pracę określam na 60%.

Podpis:

Patrycja Paciorek

Uniwersytet Łódzki

Wydział Biologii i Ochrony Środowiska

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Łódź 16/05/2017

OŚWIADCZENIE

Oświadczam, że w pracy:

*Silver nanoparticles can attenuate nitrative stress.*Żuberek M, **Paciorek P**, Bartosz G, Grzelak A

Mój udział polegał na przeprowadzeniu doświadczeń in vitro związanych z określeniem poziomu nitracji tyrozyny. Mój wkład w powyższą pracę określam na 1%.

Podpis:

Paciorek

Prof. dr hab. Grzegorz Bartosz

Wydział Biologii i Ochrony Środowiska

Katedra Biofizyki Molekularnej

Pomorska 141/143, 90-236 Łódź, Polska

Łódź 23/03/2018

OŚWIADCZENIE

Oświadczam, że w pracy:

Silver nanoparticles can attenuate nitrative stress. Mariusz Żuberek, Patrycja Paciorek, Grzegorz Bartosz, Agnieszka Grzelak

Mój udział polegał na dyskusji wyników i wkładzie w przygotowanie publikacji do druku. Udział ten oceniam na 3 %.

Podpis:

G. Bartosz

Dr Agnieszka Grzelak

Uniwersytet Łódzki

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Katedra Biofizyki Molekularnej

Banacha 12/16, 90-237 Łódź, Polska

Łódź 16/05/2017

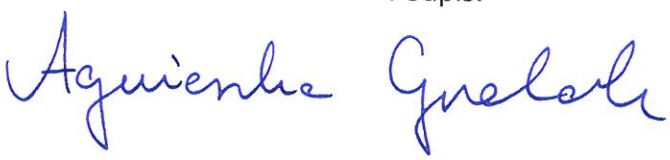
OŚWIADCZENIE

Oświadczam, że w pracy:

Silver baboparticles can attenuate nitrative stress. Zuberek M, Paciroek P., Bartosz G., **Grzelak A.**

Mój udział polegał na opracowaniu koncepcji badań, wprowadzeniu poprawek do manuskryptu i zatwierdzeniu ostatecznej wersji do złożenia. Swój udział oceniam na 36%.

Podpis:



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Łódź 16/05/2017

OŚWIADCZENIE

Oświadczam, że w pracy:

Exposure of human neurons to silver nanoparticles induces similar pattern of ABC transporters gene expression as differentiation: Study on proliferating and post-mitotic LUHMES cells. **Zuberek M**, Stępkowski TM, Kruszewski M, Grzelak A.

Mój udział polegał na oznaczeniu ilości mRNA dla wybranych genów, opracowaniu koncepcji badań, statystycznej analizie danych oraz na przygotowaniu manuskryptu publikacji. Mój wkład w powyższą pracę określam na 55%.

Podpis:

Warszawa 16/05/2017

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OŚWIADCZENIE

Oświadczam, że w pracy:

Exposure of human neurons to silver nanoparticles induces similar pattern of ABC transporters gene expression as differentiation: Study on proliferating and post-mitotic LUHMES cells Zuberek M, **Stępkowski TM**, Kruszewski M, Grzelak A.

mój udział polegał na przygotowaniu próbek z hodowli komórek LUHMES oraz na izolacji kwasów nukleinowych. Swój udział w tej pracy oceniam na 4%.

Podpis: 

Warszawa 16/05/2017

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OŚWIADCZENIE

Oświadczam, że w pracy:

Exposure of human neurons to silver nanoparticles induces similar pattern of ABC transporters gene expression as differentiation: Study on proliferating and post-mitotic LUHMES cells Zuberek M, Stępkowski TM, **Kruszewski M**, Grzelak A.

mój udział polegał na uczestniczeniu w opracowaniu koncepcji badań, wprowadzaniu poprawek do manuskryptu i zatwierdzeniu ostatecznej wersji do złożenia. Swój udział w tej pracy oceniam na 1%.

Podpis:

Dr Agnieszka Grzelak

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Podpis:

Agnieszka Grzelak