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The Interaction of Metal Nanoparticles (Copper, Silver, Platinum, and Gold) with Cell Line HS-5

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Original article

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The usage of metal nanoparticles has gained popularity due to their unique properties. However, their effect on living cells is so far, unknown. The objective of the current study was to explain the actions of copper, platinum, silver, and gold nanoparticles in respect to cell functions. In order to check the nanoparticles' features, a physicochemical analysis and morphological observation was executed using a transmission electron microscope. The viability of the cells used to detect the nanoparticles' toxicity was tested using a XTT cell proliferation assay. A scratch test confirmed the cells' ability to migrate after being treated with an increasingly larger concentration of metal nanoparticles. The results showed that silver nanoparticles are the most harmful to the HS-5 cell line in a dose-dependent manner. Additionally, copper and gold nanoparticles had the highest percentage of cell proliferation and movement ability.

Key words: migration, nanoparticles, metals, cell line HS-5.

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Nowadays, nanotechnology is one of the fastest developing areas of science with many potential benefits to human life. Using nanoparticles as a drug delivery system enhances the bioavailability of precise medication (GWINN & VALLYATHAN 2006). The influence of nanoparticles on cells is not the same for every cell type (RANA & KALAICHELVAN 2013). Although cells with a proper phenotype have not been found to be sensitive to the toxic mechanism of nanoparticles, cancer cells (for example melanoma) are. This process has pertained to copper nanoparticles (CHAKRABORTY & BASU 2017). Silver nanoparticles, the most popular among metal nanoparticles, are well-known for their toxic properties in regards to a variety of microorganisms (RANA & KALAICHELVAN 2013) but also to typical eukaryotic cells (FRANCHI *et al.* 2015). Great antioxidant properties have been ascribed to platinum nanoparticles which reduce reactive oxygen in species that interfere with a signal transduction (KONIECZNY *et al.* 2013).

The absorption of nanoparticles may occur both by inhalation and transdermally. Inside the body, the small size of nanoparticles makes it easy for them to penetrate cells causing diverse effects. They interact with the cell membrane as well as organelles and can even interact with genomes (EXBRAYAT *et al.* 2015).

The unique properties which characterize complicated structures like nanoparticles are conditioned by their high reactivity (STRAMBEANU *et al.* 2015). It has been proved that small-sized nanoparticles may be more toxic than bigger ones (ZHANG *et al.* 2014). However, many factors modify the activity of nanoparticles (also shape or colloidal stability) (PULIT *et al.* 2011; RANA & KALAICHELVAN 2013; SHANG *et al.* 2014). There is no possibility to unequivocally ascertain the toxic properties of metal nanoparticles because they are is still under examination (PULIT *et al.* 2011; LANGAUER-LEWOWICKA & PAWLAS 2014).

Cell migration fills a noteworthy part in the functioning of living organisms being the base of morphogenesis, immune response, and wound healing (KŁOPOCKA & KORCZYŃSKI 2018). Directed cell movement is a complex process that requires the coordination of several phenomena occurring in individual areas inside a cell. The principal structures associated with a cell's ability to move are the lamellipodia, located right below the cell membrane, and the microspikes (called filopodia) that project outside the cell (STĘPIEŃ *et al.* 2006). These protrusions receive environmental signals, thereby forming a leading edge in the proper direction. During this process, polymerization and shortly afterwards the contraction of actin filaments occur. It is determined by the Rho family of small G proteins due to their main role in the transmission of intracellular signals (KLIMASZEWSKA *et al.* 2011).

Regardless the nanoparticles' toxicity or their lack, they may be used as a supplement to developing treatments in which there is an emphasis on selective action on the relevant structures. As was mentioned by FRANCHI *et al.* (2015), there is a necessity to perform more research on typical normal cells to investigate the potentially toxic properties of metal nanoparticles. Normal cells could be represented by the HS-5 cell line that is a well-known biological model. It has previously been used in nanotoxicity studies by other researchers (SUBBIAH *et al.* 2015). Taking that aspect into account and the fact that it is often chosen as a control sample in studies of cancer cells (SOSNOWSKA *et al.* 2017; JAWORSKI *et al.* 2019), it is possible to rely on this model used in previous research so as to enable extended analyses of various types of diseases including other types of cells or for examining the bioaccumulation of nanoparticles in organisms. This research also provides the basis for further studies due to its *in vitro* nature and the need to examine the possible toxicity of the used substances before performing subsequent experiments, including *in vivo* analyses. The aim of the study is to provide a better understanding of the effect of metal nanoparticles on eukaryotic cells which could improve modern therapies.

Materials and Methods

In vitro model

The HS-5 cell line was obtained from the American Type Cell Collection (Manassas, VA, USA) and the cell culture was grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (Life Technologies, Houston, TX, USA), 1% penicillin, and streptomycin (Life Technologies) in dishes for adherent growth in the incubator (RS Biotech Galaxy R+, Great Britain) with a standard condition of 37°C and 5% carbon dioxide. The cell passage was performed when confluence equaled 75% depending on the size of the culture vessels.

Characterization of nanoparticles

Copper and silver nanoparticles (Cu-NPs and Ag-NPs) were obtained from aXonnite (Nano-Tech, Warsaw, Poland), gold and platinum nanoparticles (Au-NPs and Pt-NPs) from Nano-Koloid (Warsaw, Poland). All types of nanoparticles were diluted from their initial concentrations to 1; 2.5; 5 μg/ml . The size (dynamic light scattering) and stability (zeta potential) of the nanoparticles was defined using Zetasizer Nano ZS (Malvern, UK). The morphology of the nanoparticles was observed using a transmission electron microscope (TEM, JOEL, Japan).

Cell viability

Cell viability was evaluated using a Cell Proliferation Kit II (XTT) (Roche protocol, Germany). The HS-5 cells were incubated in 96-well plates (5×10^3 cells per well) with the metal nanoparticles (in concentrations of 1; 2.5; 5 μg/ml). Additionally, untreated cells were used for control groups and medium without cells served as blank probes. Each sample was prepared in triplicate. Then a XTT solution was added into each well and incubated for 3h at 37°C. The optical density (OD) was recorded using a microplate reader (Infinite M200, Tecan, Switzerland) on a wavelength of 450 nm. The results were expressed as percentage of cell viability (ODtest - ODblank)/(OD-control - ODblank), where OD test was the optical density of cells exposed to appropriate nanoparticles' type and concentration. Results were collected after incubating for 3 and 24 hours. During the test, cell morphology was also examined using a ProgRes c12 microscope camera (Jenoptik, Jena, Germany).

Cell migration and proliferation

To determine cell migration and proliferation, a scratch test was done. The HS-5 cells were maintained in DMEM in Petri dishes with a 35 millimeter diameter until they created a monolayer. Then a scratch was made using a sharp tool and the cell culture was incubated in changed conditions (2% Fetal Bovine Serum, Life Technologies, Houston, TX, USA) and the highest concentration of each metal nanoparticle. After 48 hours, the cells were stained using a May-Grünwald solution (Sigma-Aldrich, Germany). The results were analyzed with a Fiji program (version ImageJ 1.50e) by counting an average amount of cells from three areas.

Statistical analysis

Results were analyzed using a one-way variance analysis (ANOVA) with Statgraphics Centrunion (Statgraphics Technologies, The Plains, VA, USA) where statistically significant differences were assumed at $p \leq 0.05$. All data were complied with the assumption of ANOVA.

Results and Discussion

The metal nanoparticles used in the current study were of various sizes. The silver and platinum nanoparticles were bigger than the gold and copper nanoparticles (Fig. 1, Fig. 2). Although a smaller size is considered to be more toxic (ZHANG *et al.* 2014), other factors may change their behavior (PULIT *et al.* 2011; RANA & KALAICHELVAN 2013; SHANG *et al.* 2014). Au-NPs and Cu-NPs were also more unstable than the rest of tested samples. The mean values of zeta potential are presented in Table 1. Taking into consideration their physicochemical properties (a small size and a lack of colloidal stability), it can be concluded that they have a tendency to agglomerate in solutions. This was also reported in a previous study (KLOCHKOV *et al.* 2012). The agglomeration of nanoparticles may significantly contribute to cellular uptake because of their ability to embed into the cell layer to a greater extent than more dispersed nanoparticles (HALAMODA-KENZAOUI *et al.* 2017). Even though the agglomeration of nanoparticles is a challenge, after estimating the size distribution and zeta potential, it is possible to conduct toxicological research. The main reason for the agglomeration is high ionic strength which is a mainstream in culture media (ZOOK *et al.* 2011).

Table 1

Mean values of zeta potential of metal nanoparticles

Samples	Zeta potential (mV)
Cu-NPs	-6.72
Pt-NPs	-16.60
Ag-NPs	-27.80
Au-NPs	-10.60

Cell viability was detected after 3 and 24 hours of incubation (Fig. 3). The results showed that silver

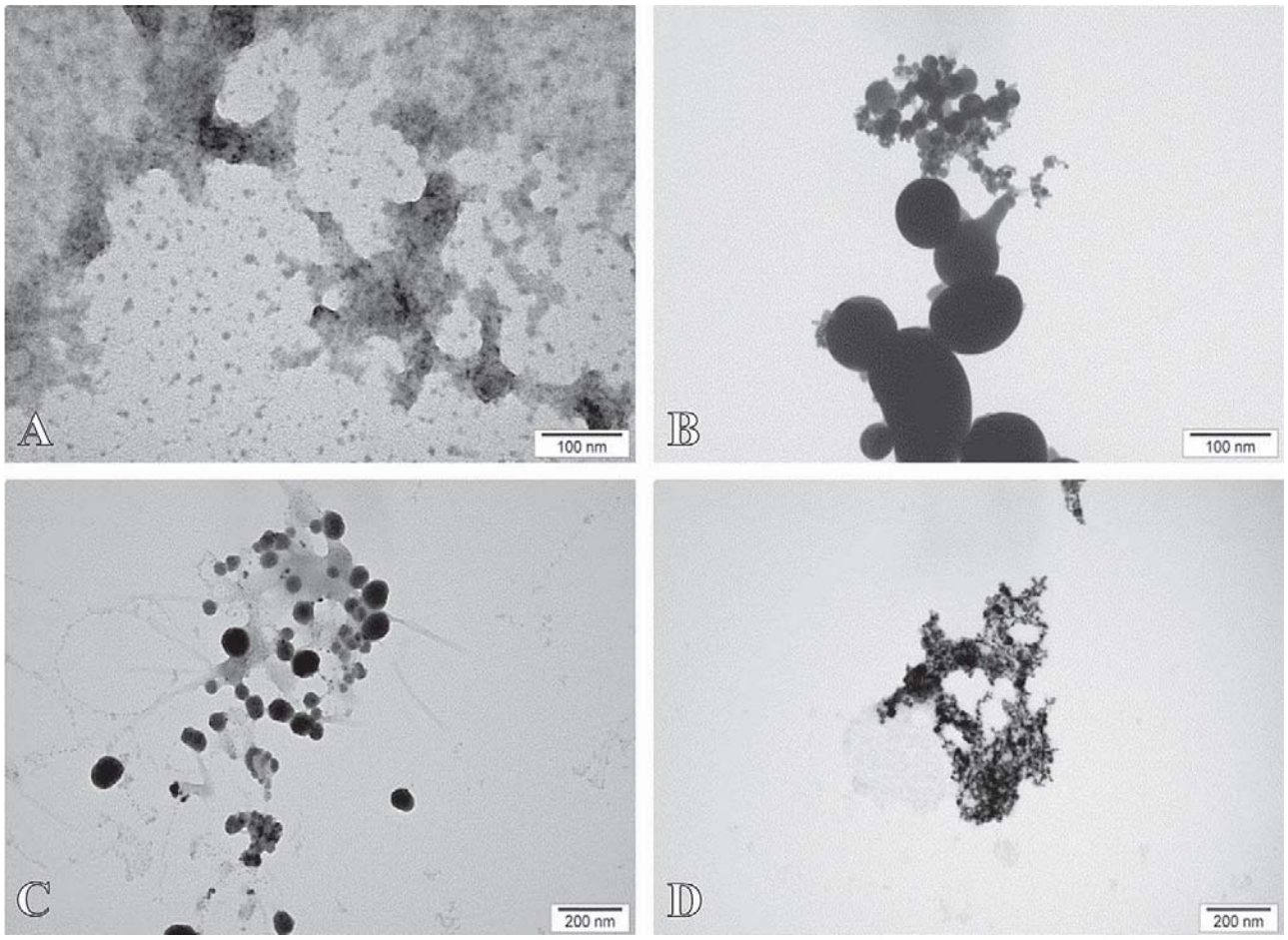

Fig. 1. Transmission electron microscopy (TEM) images of metal nanoparticles, (A) copper, (B) platinum, (C) silver, (D) gold.

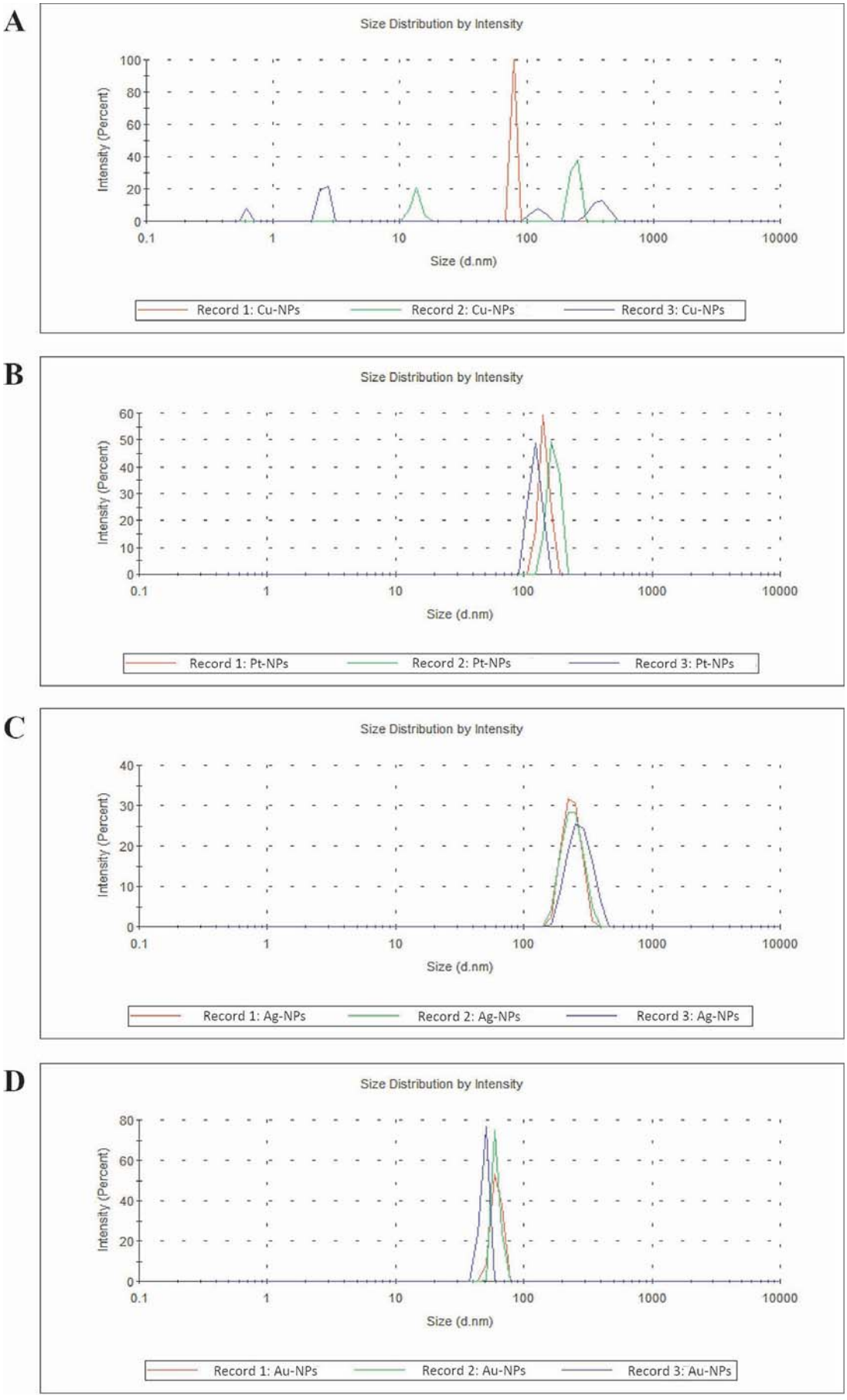

Fig. 2. Size distribution of (A) copper, (B) platinum, (C) silver, (D) gold nanoparticles measured by dynamic light scattering method.

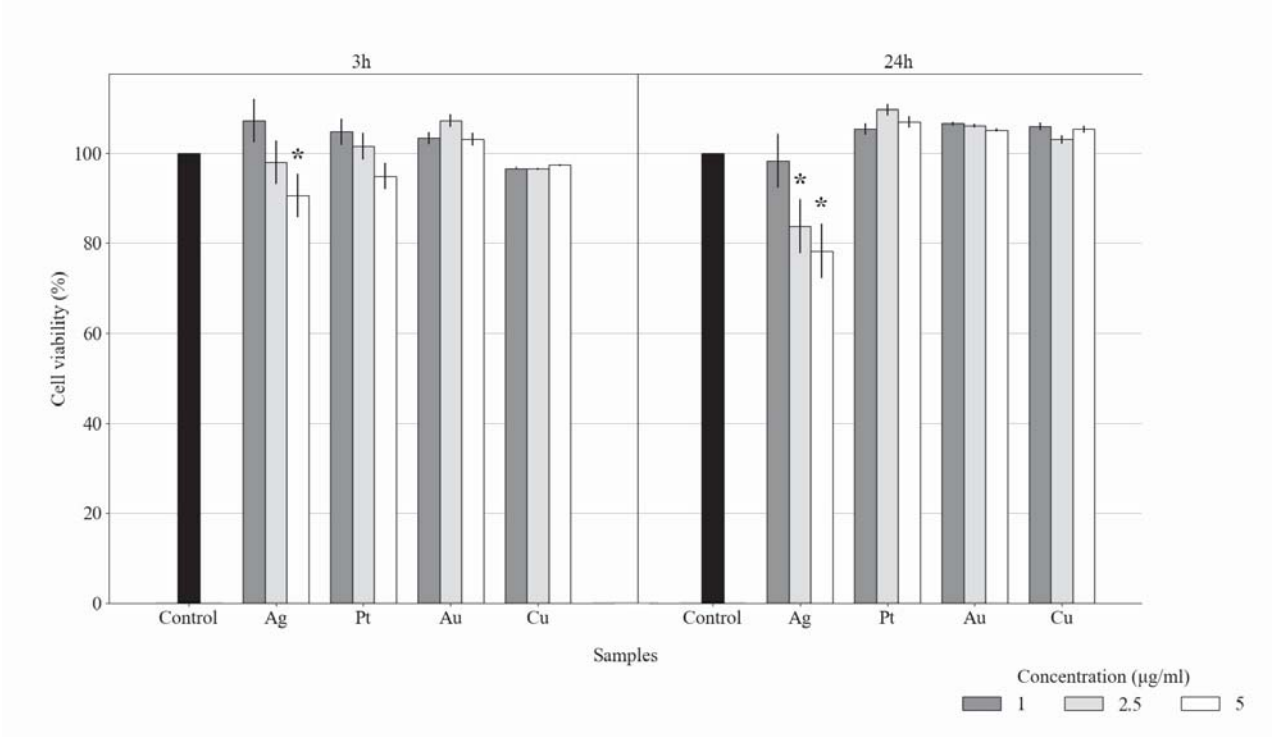

Fig. 3. Influence of metal nanoparticles on cell viability according to the incubation time. Each bar is a mean value ± standard deviation. * statistically significant differences (p-value ≤ 0.05)

nanoparticles exerted the most toxic effect on cells, increasing with extended incubation time and higher concentration. According to previous investigations, the deleterious effect of Ag-NPs on mammalian cells has already been confirmed (FRANCHI *et al.* 2015; SOOK *et al.* 2015; ZAPÓR 2016; XUE *et al.* 2018). In contrast, platinum and copper nanoparticles had an opposite effect – toxicity was lower with prolonged incubation. Platinum is widely known for its remarkable antioxidant (KONIECZNY *et al.* 2013) and catalytic properties (KSIĄŻYK *et al.* 2015; BROWN *et al.* 2018). Pt-NPs are supposed to capture reactive oxygen species even though they were previously a stress factor. Au-NPs can interact with compounds in growth media and change their properties such as changing the surface charge. If the surface is cationic, it is more toxic (ALKILANY & MURPHY 2010). In this study, the harmful effect of Au-NPs on eukaryotic cells was not proved. There is a possibility that the Au-NPs had an anionic surface, or that they interacted with the medium proteins which may have occurred because of their low colloidal stability (Table 1). We are in agreement with VIEIRA *et al.*'s (2017) research which showed that gold nanoparticles may alter cells' physiology without affecting their viability.

Gold and copper nanoparticles contributed the most of all probes to cell movement (Fig. 4). Despite recorded migration in all the samples during the 48-hour period in relation to the control, even those previously considered to be toxic, such as silver nanoparticles, contributed to increased cell migration. In Fig. 4, the movement of the cells that overgrew the previously made scratch can be observed. These cells showed a normal phenotype, although in the case of the platinum sample the cells had slightly shrunk. The untreated probe (control) did not show the highest number of migrating cells, but the migration process was evident due to visible protrusions after 48 hours.

In a previous experiment, the surface of gold nanoparticles was observed to have a significant impact on cell migration (PITCHAIMANI *et al.* 2017). The results of cell migration are shown in Table 2. The cells' morphology clearly indicated that they migrated due to the visible lamellipodia which were observed in all samples (Fig. 5). The biggest number of the dead cells was observed in the probes treated with platinum nanoparticles. Numerous and extensive protrusions were observed in the copper and gold nanoparticle probes in accordance with the previous test (the scratch test) where the same factors caused the highest percent of migration.

The lamellipodia received environmental signals which resulted in the formation of a leading edge (KLIMASZEWSKA *et al.* 2011). This implies that metal nanoparticles interact with the cell membrane. Nevertheless, this is not the only evidence pointing to the

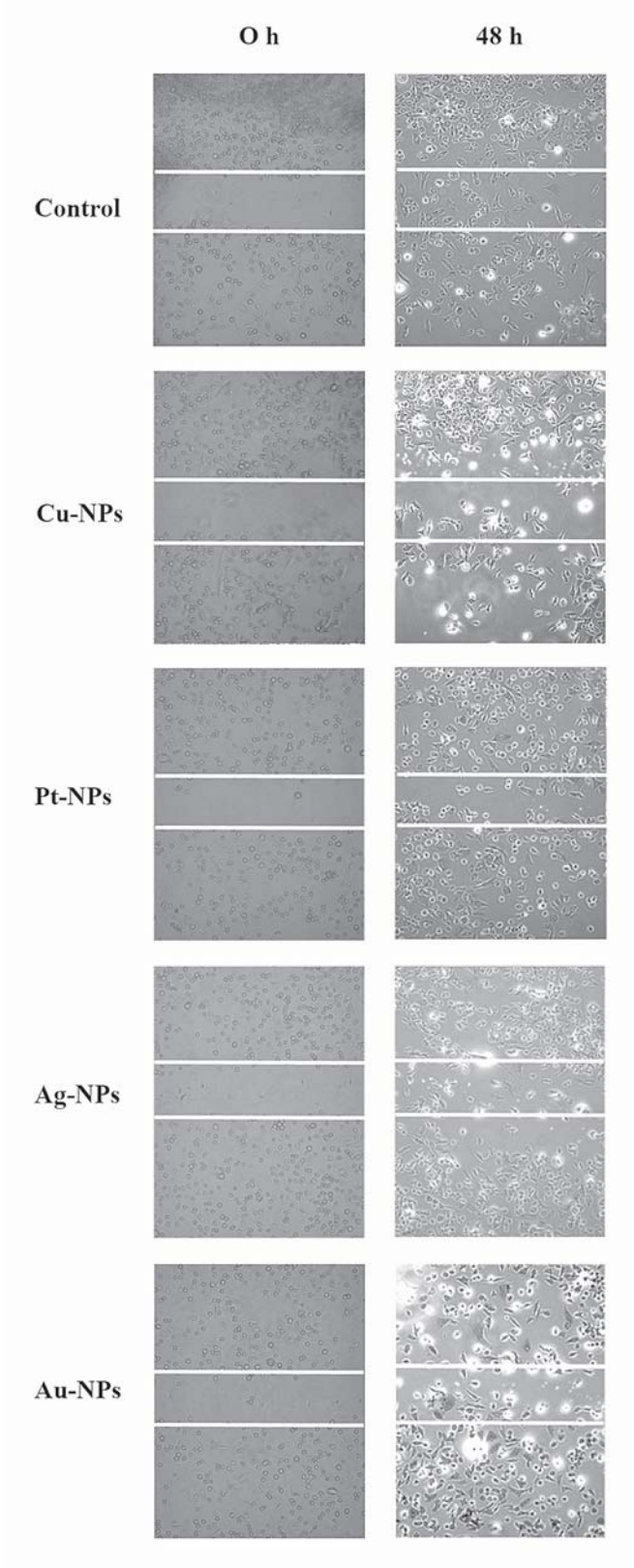

Fig. 4. Results of the scratch test showing the overgrown area (48 h) relative to the control (0 hours). Each picture showing a scratch at time 0 (0 h) was a reference for overgrowth process. All tests were performed in the presence of an untreated control; magnification 10x.

Table 2

Percentage of HS-5 cell line migration.

Samples	Migration
Cu-NPs	72%*
Pt-NPs	53%
Ag-NPs	61%
Au-NPs	68%
Control	55%

* statistically significant differences ($p \leq 0.05$)

impact nanoparticles have on cells. Before interacting with a cell membrane, NPs may be tampered with by ECM proteins which create a corona-coat. Proteins gathered around NPs, alter their properties giving them a more accessible design. The penetration of cells occurs mainly through endocytosis (it also depends on size and type). Inside the cells, their further purpose is defined in conjunction with the Golgi apparatus, endoplasmic reticulum, and with lysosomes (BEHZADI *et al.* 2017). At the cellular level, it is the Golgi apparatus that is responsible for copper homeostasis (KIOUMOURTZI 2015). Previously, the HS-5 cell line was sensitive to Cu-NPs, however, this sensitivity disappeared with prolonged time. Copper nanoparticles caused the highest percentage of migration, which suggests that unless Cu-NPs destroy internal organelles, cells will expand their secretory ability. Considering nanoparticles' ability to escape from the endoplasmic vesicle to the cytoplasm or other compartments (BEHZADI *et al.* 2017), this kind of reaction seems probable. Besides that, NPs can vary intracellular pathways affecting the Rho family (KLIMASZEWSKA *et al.* 2011). As a result, this could change the percentage of migration. The presence of dead cells may prove that nanoparticles have an influence on genomes or different organelles. It is suggested that nanoparticles can enter different parts of the body, including the bone marrow and its cells, where they have different effects depending on the type of nanoparticle (EXBRAYAT *et al.* 2015). In addition, the effect of nanoparticles on non-cancerous cells is important in determining the toxicity that nanoparticles can have on healthy tissue. This assumption was made by FRANCHI *et al.* (2015) using normal human fibroblasts for their *in vitro* studies, who at the same time underlined that there are few studies that determine the cytotoxicity of metal nanoparticles in healthy tissues.

In summary, nanoparticles may interact with cells on various levels. This depends on the unusual properties of nanoparticles as well as their activity within cells.

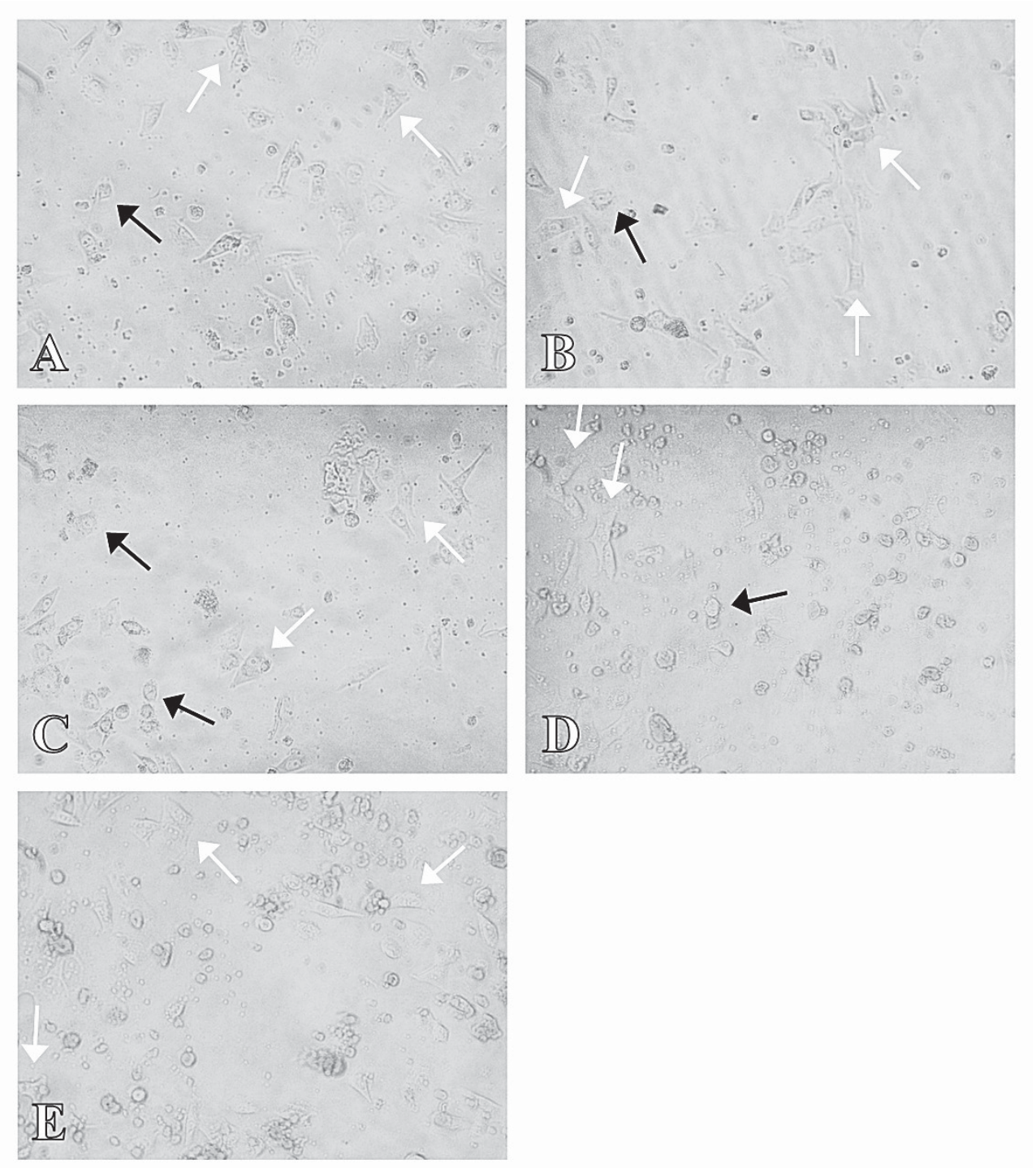

Fig. 5. Cell morphology during XTT assay, probes: (A) control, (B) copper, (C) platinum, (D) silver, (E) gold nanoparticles; white arrows point to lamellipodia, black arrows point to dead cells, magnification 40x.

Conclusion

The effect of metal nanoparticles on eukaryotic cells with a proper phenotype was confirmed. Silver nanoparticles were observed to be the most toxic with an increasing concentration from 1 to 5 μg/ml, in dose-dependent manner. Copper and gold nanoparticles significantly affected the migration of the HS-5 cell line compared to the control group. These findings give encouraging results which form the basis for future experimental science.

Author Contributions

Research concept and design: S.J.; Collection and/or assembly of data: A.L., S.J.; Data analysis and interpretation: A.L., S.J.; Writing the article: A.L., S.J., A.K.; Critical revision of the article: A.L., S.J., A.K.; Final approval of article: S.J.

Conflict of Interest

The authors declare no conflict of interest.

References

ALKILANY A.M., MURPHY C.J. 2010. Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? J. Nanopart. Res. **12**: 2313-2333. https://doi.org/10.1007/s11051-010-9911-8

BEHZADI S., SERPOOSHAN V., TAO W., HAMALY M.A., ALKAWAREEK M.Y., DREADEN E.C., BROWN D., ALKILANY A.M., FAROKHZAD O.C., MAHMOUDI M. 2017. Cellular uptake of nanoparticles: journey inside the cell. Chem. Soc. Rev. **46**: 4218-4244. https://doi.org/10.1039/c6cs00636a

BROWN A.L., KAI M.P., DUROSS A.N., SAHAY G., SUN C. 2018. Biodistribution and Toxicity of Micellar Platinum Nanoparticles in Mice via Intravenous Administration. Nanomaterials **8**: 410. https://doi.org/10.3390/nano8060410

CHAKRABORTY R., BASU T. 2017. Metallic copper nanoparticles induce apoptosis in a human skin melanoma A-375 cell line. Nanotechnology **28**: 105101. doi:10.1088/1361-6528/aa57b0

EXBRAYAT J.M., MOUDILOU E.N., LAPIED E. 2015. Harmful Effects of Nanoparticles on Animals. J. Nanotechnol. 2015: 1-10. https://doi.org/10.1155/2015/861092

FRANCHI L., MANSHIAN B., DE SOUZA T., SOENEN S., MATSUBARA E., ROSOLEN J., TAKAHASHI C. 2015. Cyto- and genotoxic effects of metallic nanoparticles in untransformed human fibroblast. Toxicology In Vitro **29**: 1319-1331. https://doi.org/10.1016/j.tiv.2015.05.010

GWINN M.R., VALLYATHAN V. 2006. Nanoparticles: Health Effects – Pros and Cons. Environ. Health Perspect. **114**: 1818-1825. https://doi.org/10.1289/ehp.8871

HALAMODA-KENZAOUI B., CERIDONO M., URBÁN P., BOGNI A., PONTI J., GIORIA S., KINSNER-OVASKAINEN A. 2017. The agglomeration state of nanoparticles can influence the mechanism of their cellular internalisation. J. Nanobiotechnol. **15**, 48. https://doi.org/10.1186/s12951-017-0281-6

JAWORSKI S., STROJNY B., SAWOSZ E., WIERZBICKI M., GRODZIK M., KUTWIN M., DANILUK K., CHWALIBOG A. 2019. degradation of mitochondria and oxidative stress as the main mechanism of toxicity of pristine graphene on U87 glioblastoma cells and tumors and HS-5 cells. Int. J. Mol. Sci. **20**: 650. https://doi.org/10.3390/ijms20030650

KIOUMOURTZI G. 2015. Heavy metal toxicity in human lung fibroblasts and inhibition of human topoisomerase-I as a potential mechanism. Master thesis, Lancaster, University of Lancaster, pp. 30

KLIMASZEWSKA A., STENZEL A., NOWAK J.M., GRZANKA A. 2011. Białka Rho – kluczowe regulatory cytoszkieletu w przebiegu mitozy i cytokinezy [Rho proteins – the key regulators of cytoskeleton in the progression of mitosis and cytokinesis] Postepy Hig Med Dosw **65**: 704-713 (In Polish with English summary). https://doi.org/10.5604/17322693.966197

KLOCHKOV V.K., GRIGOROVA A.V., SEDYH O.O., MALYUKIN Y.V. 2012. The influence of agglomeration of nanoparticles on their superoxide dismutase-mimetic activity. Colloids and Surfaces A: Physicochemical and Engineering Aspects **409**: 176-182. https://doi.org/10.1016/j.colsurfa.2012.06.019

KŁOPOCKA W., KORCZYŃSKI J. 2018. Migracja – regulacja zjawiska przez wybrane szlaki sygnałowe [Migration, mechanisms and regulation principles]. Kosmos – problemy nauk biologicznych, **67**: 207-218. (In Polish with English summary).

KONIECZNY P., GORALCZYK A.G., SZMYD R., SKALNIAK Ł., KOZIEL J., FILON F.L., CROSERA M., CIERNIAK A., ZUBA-SURMA E. K., BOROWCZYK J., LACZNA E., DRUKALA J., PYZA E., SEMIK D., WOZNICKA O., KLEIN A., JURA J. 2013. Effects triggered by platinum nanoparticles on primary keratinocytes. Int. J. Nanomed. **8**: 3963-3975. https://doi.org/10.2147/IJN.S49612

KSIĄŻYK M., ASZTEMBORSKA M., STĘBOROWSKI R., BYSTRZEJEWSKA-PIOTROWSKA G. 2015. Toxic Effect of Silver and Platinum Nanoparticles Toward the Freshwater Microalga Pseudokirchneriella subcapitata. Bull. Environ. Contam. Toxicol. **94**: 554-558. https://doi.org/10.1007/s00128-015-1505-9

LANGAUER-LEWOWICKA H., PAWLAS K. 2014. Nanocząstki, nanotechnologia – potencjalne zagrożenia środowiskowe i zawodowe [Nanoparticles, nanotechnology – potential environmental and occupational hazards]. Med. Srod. **17**: 7-14. (In Polish with English summary).

PITCHAIMANI A., NGUYEN T.D.T., KOIRALA M., ZHANG Y., ARYAL S. 2017. Impact of cell adhesion and migration on nanoparticle uptake and cellular toxicity. Toxicol. in Vitro **43**: 29-39. https://doi.org/10.1016/j.tiv.2017.05.020

PULIT J., BANACH M., KOWALSKI Z. 2011: Właściwości nanocząsteczek miedzi, platyny, srebra, złota i palladu [Properties of nanoparticles of copper, platinum, silver, gold, and palladium]. Czasopismo Techniczne. Chemia **108**: 197-209 (In Polish with English summary).

RANA S., KALAICHELVAN P.T. 2013. Ecotoxicity of Nanoparticles. ISRN Toxicology: 1-11. https://doi.org/10.1155/2013/574648

SOOK F., WAGNER S., STAHL F., KHAYDAROV R.R., SCHEPER T., BAHNEMANN D. 2015. Investigations of the Toxic Effect of Silver Nanoparticles on Mammalian Cell Lines. J. Nanomat., 2015: 1-9. https://doi.org/10.1155/2015/136765

SHANG L., NIENHAUS K., NIENHAUS G.U. 2014: Engineered nanoparticles interacting with cells: size matters. J. Nanobiotechnology **12**: 1-11. https://doi.org/10.1186/1477-3155-12-5

SOSNOWSKA M., SAWOSZ E., KUTWIN M., CHWALIBOG A. 2017. Carbon nanoscaffolds for fibroblast and hepatocellular carcinoma cells adhesion, migration and regeneration. Engineering of Biomaterials **20**: 58.

STĘPIEŃ A., GRZANKA A., SZPECHCIŃSKI A. 2006. Struktury cytoszkieletu aktynowego formowane przy krawędzi wiodącej komórki podczas pierwszego etapu migracji [Cytoskeletal actin structures formed at the leading edge of the migrating cell during the first step of migration]. Postępy Biologii Komórki **33**: 381-392 (In Polish with English summary).

STRAMBEANU N., DEMETROVICI L., DRAGOS D., LUNGU M. 2015. Nanoparticles: Definition, Classification and General Physical Properties. (In: Nanoparticles' Promises and Risks. LUNGU M., NECULAE A., BUNOIU M., BIRIS C. eds. Springer, Cham): 3-8. https://doi.org/10.1007/978-3-319-11728-7_1

SUBBIAH R., JEON S.B., PARK K., AHN S.J., YUN K. 2015. Investigation of cellular responses upon interaction with silver nanoparticles. Int. J. Nanomedicine **10**: 191-201. https://doi.org/10.2147/IJN.S88508

VIEIRA L.F. de A., LINS M.P., VIANA I.M.M.N., DOS SANTOS J.E., SMANIOTTO S., REIS M.D. DOS S. 2017. Metallic nanoparticles reduce the migration of human fibroblasts *in vitro*. Nanoscale Res. Lett. **12**: 200. https://doi.org/10.1186/s11671-017-1982-3

XUE Y., WANG J., HUANG Y., GAO X., KONG L., ZHANG T., TANG M. 2018. Comparative cytotoxicity and apoptotic pathways induced by nanosilver in human liver HepG2 and L02 cells. Hum. Exp. Toxicol. **37**: 1293-1309. https://doi.org/10.1177/0960327118769718

ZAPÓR L. 2016. Effects of silver nanoparticles of different sizes on cytotoxicity and oxygen metabolism disorders in both reproductive and respiratory system cells. Arch. Environ. Prot. **42**: 32-47. https://doi.org/10.1515/aep-2016-0038

ZHANG T., WANG L., CHEN Q., CHE C. 2014. Cytotoxic Potential of Silver Nanoparticles. Yonsei Med. J. **55**: 283-291. https://doi.org/10.3349/ymj.2014.55.2.283

ZOOK J. M., MACCUSPIE R.I., LOCASCIO L.E., HALTER M.D., ELLIOTT J.T. 2011. Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity. Nanotoxicology ***5***: 517-530. https://doi.org/10.3109/17435390.2010.536615