Annals of Warsaw University of Life Sciences – SGGW
Animal Science No 56 (1), 2017: 43–51
(Ann. Warsaw Univ. of Life Sci. – SGGW, Anim. Sci. 56 (1), 2017)
DOI 10.22630/AAS.2017.56.1.6

Influence of melittin on viability and integrity of cell membrane on grade IV glioma

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Abstract: *Influence of melittin on viability and integrity of cell membrane on grade IV glioma.* The grade IV glioma is one of the malignant human tumours. Today there is no effective treatment for this type of cancer. Alternative methods are sought-after in glioma treatment, and lately melittin has been found to be useful in anticancer therapy. The aim of the study was to investigate the effect of melittin on the viability and the integrity of cell membranes of the grade IV glioma cells. The U87 glioma line cells were treated of melittin in increasing concentrations (5, 10, 15, 20 and 50 μg/mL) and incubated for 24 h. After incubation, the tests were performed in order to investigate the cell morphology, cell viability, membrane integrity and the way of cell death. The results have shown the devastating effect of melittin on the glioma cells. The melittin causes disintegration of cell membranes and induces cell death by apoptosis and less by necrosis.

Key words: glioma, melittin, toxicity, apoptosis

INTRODUCTION

Melittin is the main toxic element of the bee poison. It is composed of 26-amino acid and is an alfa-helical polypeptide with amphipathic character (C-end is hydrophilic and N-end is hydrophobic) (Raghuraman and Chattopadhyay 2007). The peptide has hemolytic proprieties and attacks every one of lipid membranes (Hoskin et al. 2008). Effects of the polypeptide consist of the destruction of lipid membrane on the physical and chemical level (Lee et al. 2004).

The interaction between melittin and lipid membranes is possible by the exchange of melittin conformation. Four alfa-helical monomers connect to each other and form tetramers. The form of tetramers inside cell membranes creates the ion channels which modify cell membranes permeability (Tosteson and Tosteson 1981). Initially, melittin monomers stick parallel to the lipid membrane then, as tetramers, change the location on the perpendicular and made pores inside the cell membranes, leading to the cell death.

Melittin also activates phospholipase and calmodulin by facilitating phospholipase A_2 (Mollay et al. 1974) and bonds degradation inside lipid membranes (Keith et al. 2010). Traditional medicine used the melittin against many diseases like rheumatism, chronic infections, even cancer. Some research has shown that major element of the bee venom, melittin, has also anti-

microbial activities. Many studies have demonstrated that melittin has strong anticancer activity (Gajski and Garaj-Vrhovac 2013). The study conducted on the colon, gastric, lung, prostate and ovarian cancer cells gave satisfactory results (Huh et al. 2012, Jo et al. 2012, Park et al. 2012, Zheng et al. 2015, Kong et al. 2016).

Grade IV glioma is a malignant brain tumour which comes from glial cells (www.abta.org). According to World Health Organization (WHO), it has the highest grade of malice. It is the most common malignant brain tumour and also one of the most mortal human tumours. Most-ly, treatment of glioma begins with surgery, but this is a difficult and risky procedure. This tumour can revive from the single cells, so innovative therapies are needed to treat glioma. In 2013 published research suggested that melittin may be useful in the anticancer treatment (Shin et al. 2013). Melittin as a factor perforating the cell membrane may cause damage to the cell membranes of grade IV glioma. The aim of the study was to investigate the effect of melittin on the viability and the integrity of the outer cell membranes and the mitochondrial membranes using the cell line U87 as a model.

MATERIAL AND METHODS

Cell cultures

Human glioma cell line U87 and was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's culture medium containing 10% fetal bovine serum (Life Technologies, Houston, TX, USA), 1% penicillin and streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in a NuAire DH Auto Flow CO_2 Air-Jacketed Incubator (Plymouth, MN, USA).

Preparation of melittin

The melittin was obtained from Sigma Aldrich (Munich, Germany). The melittin powder was solubilising in ultrapure water to prepare stock 1.0 mg/mL solution. The solution was diluted to different concentrations (5, 10, 15, 20, 50 mg/L) with 1× Dulbecco's modified Eagle's culture medium (Life Technologies, Houston, TX, USA) immediately prior to exposure to the cells.

Cell morphology

U87 cells were plated in six-well plates (1×10^5 cells per well) and incubated for 24 h. Cells cultured in medium without the addition of melittin were used as the control. Melittin was introduced to the cells at increasing concentrations (5, 10, 15, 20 and 50 μg/mL). The same procedure was repeated during following tests. Cell morphology was recorded using an optical microscope (Olympus CKX4, Poland) at 24-hour post-exposure.

Cell viability

Cell viability was evaluated using PrestoBlue® Cell Viability Assay (Life Technologies, Taastrup, Denmark). The reagent added to cells, is reduced by metabolically active cells and turns red in colour, allowing a quick measure of viability and cytotoxicity. U87 cells were incubated on 96-well plates (5×10^3 cells per well) for 24 h. After incubation, the medium was removed and melittin

solutions (5, 10, 15, 20 and 50 μg/mL) were added to the wells at increasing concentration (90 μl per well) and the plates were incubated for an additional 24 h. Cells incubated without melittin were used as a control group. In the next step, 10 μl of PrestoBlue™ Reagent was added directly to cells in culture medium and incubated for 2 h at 37°C. After incubation the optical density of each well was recorded at 570 nm on an enzyme-linked immunosorbent assay reader (Infinite M200, Tecan, Durham, NC, USA). Cell viability was expressed as the percentage (OD-test × 100%) / / (OD-control, where "OD-test" is the optical density of cells exposed to melittin and "OD-control" is the optical density of the control sample.

Membrane integrity

A lactic dehydrogenase (LDH) test (LDH-based in vitro toxicology assay kit, Sigma-Aldrich) was used to evaluate cell membrane integrity. This test is based on enzymatic reactions resulting in a coloured product determined spectrophotometrically. U87 cells were plated in 96-well plates (5×10^3 cells per well) and incubated for 24 h. In the next step, the medium was removed and melittin solutions were added to the cells at increasing concentration. After 24 h of incubation, half of the volume of the culture medium was removed and added LDH assay mixture. A final volume for LDH assay was a 100 μl per well. The plate was incubated for 20 min without light at room temperature. The OD was detected as outlined and the LDH leakage was expressed as the percentage of OD using a formula: 100 – (OD-control / / OD-test) × 100%.

Mitochondrial transmembrane potential

Mitochondrial Transmembrane Potential Apoptosis Detection Kit (Abcam, Cambridge, UK) was used to test mitochondrial transmembrane potential as an indicator of cell death. In this test U87 glioma cells were plated in six-well plates (1×10^5 cells per well) and incubated for 24 h. In the next step, the medium was removed and melittin solutions were added to the cells at increasing concentration and were incubated for 24 h. Cells cultured in medium without the addition of melittin were used as the control. The cells were harvested and suspended in 1 ml of the diluted MitoCapture solution. After 20 min of incubation with the reagent in 37°C and 5% CO_2 mitochondrial permeability was analysed using fluorescence microscope (Olympus CKX41, Poland) 24 h after exposure. MitoCapture that has aggregated in the mitochondria of healthy cells fluoresces red, MitoCapture cannot accumulate in mitochondria, it remains as a monomer in the cytoplasm, and fluoresces green.

Apoptosis/necrosis assay

An annexin V/PI assay (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit with AlexaFluor 488 Annexin V and propidium iodide (PI) for flow cytometry, Life Technologies) was performed to test way of cells death. After 24 h incubation of U87 glioma cells in 75 ml flasks (1×10^6 cells per flask), the medium was removed, and melittin solutions were added at 20 μg/mL. After further 24 h incubation, the medium was removed and the cells were washed in

ice-cold PBS. Harvested cells were suspended in 100 µl annexin-binding buffer (Invitrogen, Carlsbad, CA, USA) and subsequently, 5 µl of annexin V linked with Alexa Fluor 488 and 1 µl of PI were added (Invitrogen, Carlsbad, CA, USA). Cells were analysed using FACStrak (Becton-Dickinson, Germany; software – SimulSet), measuring the fluorescence emission at 530 nm and 575 using excitation at 488 nm.

Statistical analysis

Data were analysed using multifactorial analysis of variance with Statgraphics® Plus 4.1 (StatPoint Technologies, Warrenton, VA, USA). The differences between groups were tested using Tukey's multiple range tests. All mean values are presented with the standard deviation.

RESULTS AND DISCUSSION

In this study, we evaluated the influence of melittin on the morphology, viability, and cell membrane integrity of glioblastoma cells. Additionally, apoptosis and necrosis were evaluated. Glioma cells were treated with melittin in increasing concentration (5, 10, 15, 20 and 50 µg/mL). Figure 1 shows representative images from the optical microscope. We observed a significant difference between control group and cells treated with 15 µg/mL. After incubation with melittin U87 glioma cells changed their morphology. Their protrusions were thinner in comparison with the control cells. Moreover, they collapsed similarly to ovarian cancer cells (Bei et al. 2015). However, they used lower concentration (3 µM ~ 8.5 µg/mL) of melittin, thus glioma cells are probably more resistant to bee venom influence.

Zhang et al. (2014) suggested that melittin has a toxic influence in higher concentration on cancer cells and toxicity is dose-dependent. Increased concentrations of melittin resulted in decreased vitality on glioma cells. The viability was reduced to 51.6% followed by 15 µg/mL of melittin and the lowest viability was observed at the concentration of 50 µg/mL, i.e. 19.5% (Fig. 2). However, the difference between control and treated cells was significant at 10 µg/mL. Melittin due to the ability to form pores in lipid membranes can perforate a cell membrane of grade IV glioma. Cells whose integrity of outer membrane was interrupted may be directed to the death or have reduced metabolic activity.

Previous studies proved that melittin inhibits proliferation of human glioma cells and causes cell death in high concentration (Yang et al. 2007). In this study, membrane integrity was monitored by LDH assay. Melittin destabilizes cell membrane functionality and integrity, and there were significant differences between melittin-treated groups (Fig. 3). Melittin had the highest toxicity at concentrations of 20 and 50 µg/mL, with membrane disintegration of 72 and 67% respectively. Several studies show that melittin has the ability to perforation of lipid membranes and our results are consistent with this. Melittin also destroys the inner cell membranes, especially mitochondrial (Dombrowski et al. 2012).

The pictures from the fluorescence microscope (Fig. 4) show that control group has a lot of active mitochondria what is seen as lighter areas, marked letter R. It corresponds to the red colour on the fluorescence microscope images.

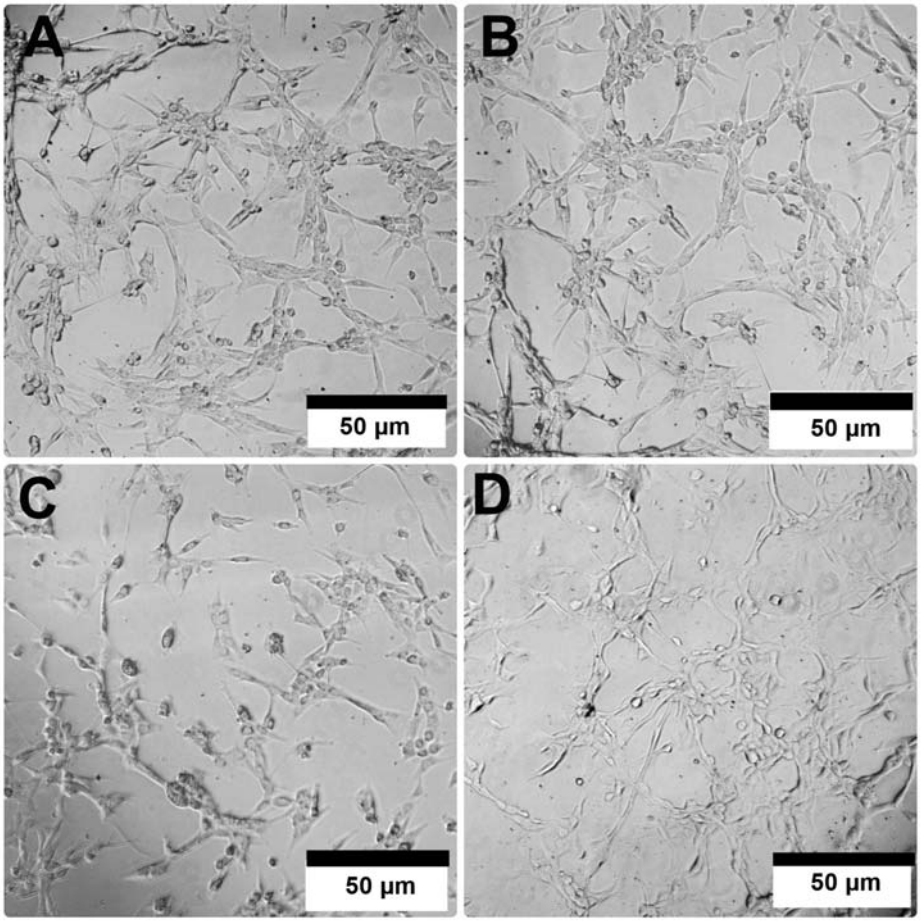

FIGURE 1. Optical microscopy images of melittin-treated and untreated U87 glioma cells. A – control; B – cells treated 5 µg/mL of melittin; C – cells treated 15 µg/mL of melittin; D – cells treated 50 µg/mL of melittin

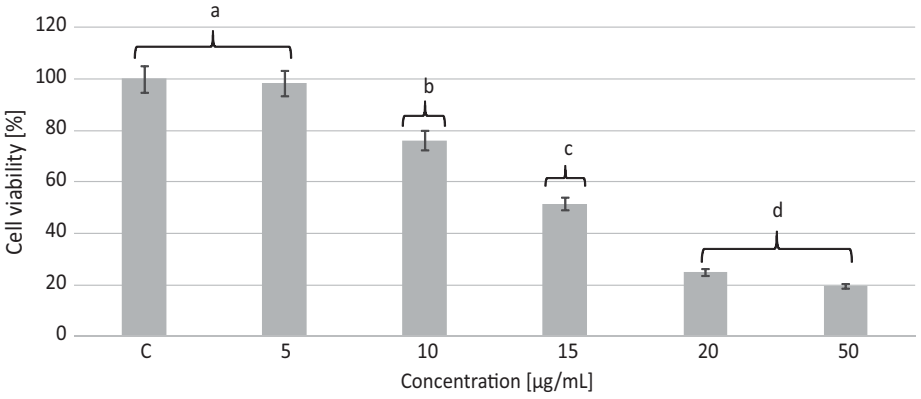

FIGURE 2. Effect of melittin, on the viability of U87 glioma cells. C – untreated cells, control group. The columns with different letters (a–d) indicate significant differences between groups (P <0.001)

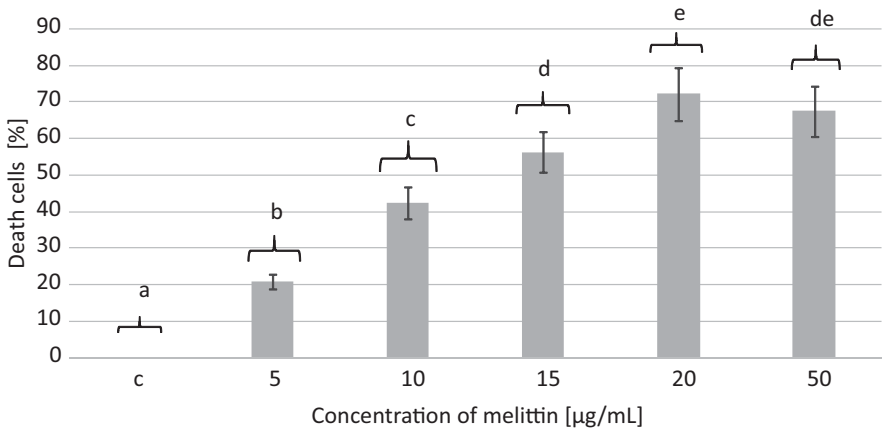

FIGURE 3. Effect of melittin on the mortality of U87 glioma cells. C – untreated cells, control group. The columns with different letters (a–e) indicate significant differences between groups (P <0.001)

The melittin-treated cells have much less active mitochondria. On the Figure 4b are noticeable mainly darker areas, marked letter G, corresponding to the green colour on the fluorescence microscopy. As melittin has the ability to perforation of mitochondrial membranes, it may induce the intrinsic apoptosis pathway, however, the cancer cells are the cell type in which very rarely death occurs by apoptosis (Wong 2011). This constitutes one of the main problems in the design of anticancer therapy. Kong et al. (2016) reported that melittin induces apoptosis in human gastric cancer cell. Jo et al. (2012) proved that melittin-induced death by apoptosis in ovarian cancer cells. In this study, the way of glioma cell death was examined by melittin. The previous studies showed that melittin induces apoptosis by intrinsic pathway (Kong et al. 2016). We received the similar results using a flow cytometer. Figure 5 shows results from the apoptosis assay. Melittin induced apoptosis to a substantial degree in U87 glioma cells (60%). The degree of necrosis was 22% only. This is a desirable effect because necrosis induces inflammation, which is not present during apoptosis (Stępień 2007). Apoptosis has two well-known pathways: intrinsic and extrinsic. The mechanism of interactions of melittin with cell membrane may induce the outer pathway of apoptosis (LDH assay). However, our research shows that mitochondrial pathway is passible too (Fig. 4). On the picture from fluorescence microscope, the control group has a lot of active mitochondria what is seen as lighter areas, marked letter R. It corresponds to the red colour on the fluorescence microscope images. The melittin-treated cells have much less active mitochondria. On the Figure 4b are noticeable mainly darker areas, marked letter G, corresponding to the green colour on the fluorescence microscopy. The mitochondrial transmembrane potential assay showed that melittin disintegrates mitochondrial membranes, which may indicate that melittin induces apoptosis by a mito-

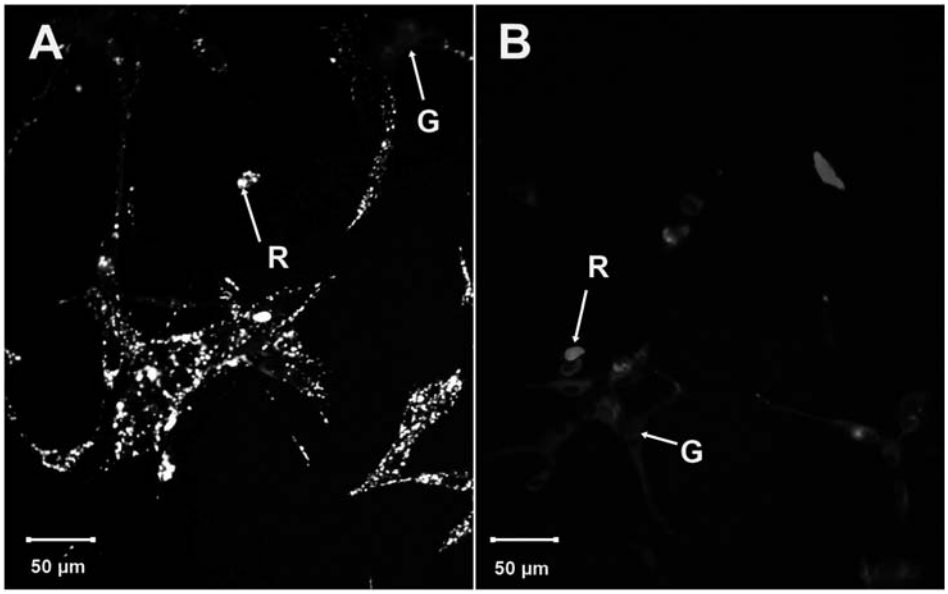

FIGURE 4. Effect of melittin on the mitochondrial membranes integrity. A – control group, untreated cells; B – cells treated 20 µg/mL of melittin (explanations in the text)

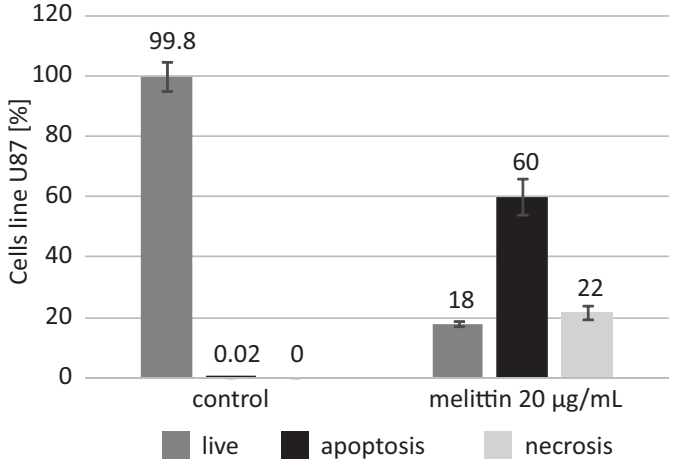

FIGURE 5. Annexin V-Alexa Fluor® 488 and PI assay analysis. Diagrams of cells exposed to 20 µL/mL of melittin

chondrial pathway in glioma cells, but to know detailed way of glioma cell death by melittin more tests are needed.

To sum up, melittin has a harmful influence on human glioma cells. By the ability to cell membrane perforation kills cancer cells by apoptosis mostly. This fact is important because the risk of inflammation is reduced. Melittin interaction with cell membranes could be exploited

for anticancer therapy. However, we need more information about the effects of this compound on the level of proteins, genes and enzymes. The present results are preliminary and follow-up research to elucidate the molecular mechanisms involved in the interactions between melittin and cells is necessary. At this stage, the results indicate that the contact between melittin and U87 cell membranes may be the key cause of melittin toxicity. Consequently, the morphology of glioma cells, the molecular status of cell lines, and the melittin concentration may act together to regulate the biological response of glioma cells to melittin.

CONCLUSION

The results demonstrate that cytotoxicity of melittin on glioma cells increases with increasing melittin concentrations from 5 to 50 μg/mL. Melittin causes perforation of cell membranes and mitochondrial membranes. However, melittin induces apoptosis and to less extent necrosis in the U87 cell line, indicating that melittin is a promising candidate for anticancer therapy.

Acknowledgement

Grant NCN PRELUDIUM 2013/09/N/ /N29/01898.

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Streszczenie: *Wpływ melityny na żywotność i integralność błon komórkowych glejaka IV stopnia.* Glejak IV stopnia jest jednym ze złośliwych nowotworów ludzkich. Do dziś nie wynaleziono skutecznego leku do walki z tym nowotworem. Melityna to jeden ze składników jadu pszczelego, który wykazuje działania antyrakowe. Celem badań było zbadanie wpływu melityny na żywotność i integralność błon komórkowych glejaka IV stopnia linii U87. Komórki glejaka IV stopnia linii U87 były traktowane roztworami melityny we wzrastającym stężeniu (5, 10, 15, 20 i 50 µg/mL) i inkubowane przed 24 h. Po inkubacji przeprowadzono badania w celu sprawdzenia morfologii komórek, ich żywotności, integralności błon komórkowych oraz drogę śmierci komórkowej. Wyniki wskazują na niszczący wpływ melityny na komórki glejaka. Melityna powoduje dezintegrację błon komórkowych oraz indukuje śmierć komórkową na drodze apoptozy oraz, w mniejszym stopniu, nekrozy.

Słowa kluczowe: glejak, melityna, toksyczność, apoptoza

MS received 10.10.2016
MS accepted 23.03.2017

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