INTRODUCTION

Obesity is a risk factor for many cancers and obese cancer patients have a poorer prognosis (1). A positive association between body mass index (BMI), visceral obesity and risk for gastrointestinal cancers is well documented (2-4). Colorectal cancer (CRC) is the third leading cause of cancer related mortality in USA and Europe (5). A number of mechanisms have been proposed for the adverse effect of obesity on colorectal cancer risk including the distribution of body fat, level of obesity related inflammation and oxidative stress, metabolic disturbances as well as alternation in fat derived hormonal patterns (6). Oxidative stress is also considered as a probable mechanism in the pathogenesis and development of many diseases, including obesity and cancer (7).

White adipose tissue (WAT) is currently recognized as an important endocrine organ. The physiological functions of adipose tissue are changed in obesity, leading to an altered secretion of adipocytokines such as: visfatin, leptin, adiponectin, resistin, which may influence cancer pathogenesis and progression (8). These adipokines may significantly influence the growth and proliferation of tumor stroma and malignant cells within primary tumor (9). Adipose tissue represents an important source of ROS, which may contribute to the development of obesity-associated cancer. The increased levels of systemic oxidative stress that occur in obesity may contribute to the obesity-associated development of the insulin resistance and type 2 diabetes, hypertension, atherosclerosis, and cancer (7, 10).

Visfatin/PBEF/Nampt is predominantly expressed in visceral and subcutaneous fat (11). This protein is a recently described as an adipocytokine with diverse and complex functions. Initially named PBEF (pre-B cell colony enhancing factor) (12), it was later found to have similarities with the nicotinamide phosphoribosilotransferase _nadh_ gene from prokaryote.
**Haemophilus ducr eyi, Nampt**

Serum levels of visfatin are elevated in subjects with visceral fat.

Visfatin stimulates glucose uptake by adipocyte and muscle cells *in vitro* and decreases blood glucose levels in mice (15). Visfatin/Nampt expression promotes cell growth and survival, angiogenesis, what is more it is highly expressed in gastric and colorectal carcinomas as well as malignant glioblastomas (16-20). It is more highly expressed in gastric and colorectal carcinomas (21).

**Visfatin/Nampt** is also highly expressed in malignant glioblastomas (16-20). It is more highly expressed in gastric and colorectal carcinomas (21).

Epidemiological studies also revealed that visfatin/Nampt levels in human serum may be a good biomarker of colorectal malignant potential, independently from BMI, and also stage of progression of colorectal (22) and gastric cancer patients (17).

Visfatin/Nampt also induces ROS-reactive oxygen species generation in human umbilical endothelial cells (23), differentiated mice myotubes (24) as well as human malignant Me45 melanoma cells (25) *in vitro*. Recently, Wang *et al.* reported that visfatin had no anti-apoptotic effect on normal cultured VSMCs, and it exerted an anti-apoptotic effect only during cell apoptosis induced by H2O2 (26). Our previous study also revealed protective role of visfatin in hydrogen peroxide-induced DNA damage in human melanoma Me45 cells (27). Recently, Jacques *et al.*, demonstrated that visfatin induced apoptosis in murine articular chondrocytes cultured *in vitro* through unknown pathway (28).

Various metabolic pathways produce ROS, including aerobic metabolism in the mitochondrial respiratory chain. It plays a critical role in the initiation and progression of various types of cancers. ROS affect different signaling pathways, including growth factors and mitogenic pathways, they control many cellular processes, such as cell proliferation and apoptosis (29). Investigation of effects of higher level of intracellular ROS in cancer cells showed the occurrence of temporary growth arrest, senescence, apoptosis or necrosis (30). Apoptosis may be connected to generation of hydrogen peroxide (H2O2) and caspase activation, while necrosis is attributed to reduced ATP level and energy failure in conjunction with thiol depletion (31). The primary defenses of normal and cancer cells against oxidative stress include antioxidant enzymes such as: superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).

Using cell model, we tried to verify the hypothesis if visfatin/Nampt derived from fat tissues influences on cell apoptosis/necrosis cells in culture upon visfatin/Nampt at different concentrations (10, 100, 250 ng/ml) and cultured with McCoy's 5A modified medium (Sigma-Aldrich, North Androver, Mass), and antibiotics (0.1 mg/mL streptomycin, 100 U/mL penicillin; PAA Laboratories), as well as: fungicide; amphotericin B (2.5 µg/ml); (P AA Laboratories). Cell cultures were led on standard sterile condition, under an atmosphere of 95% air and 5% CO2 at 37°C in HERAcell incubator (Thermo scientific). Afterwards, cells were harvested by trypsinization, and the viable cells were counted in an automated cell counter (model no. TC 20; Bio-Rad) using 0.4% trypan blue solution. The amount of dead cells in cell culture did not exceed 4%. HCT 116 cell line was free of bacteria species, mycoplasma and yeast-like fungi. Cultures were maintained for no longer than four weeks after recovery from frozen stocks. Fungizone was added to cell culture but only in the first passage of cells obtained from frozen stock, functional tests were assayed without fungicide.

**Drug preparation and treatment regimens**

Visfatin/Nampt (Enzo Life-Science, Plymouth, PA, USA) was dissolved in phosphate buffered saline PBS, without Mg2+, Ca2+ (Sigma-Aldrich, St Louis, Mo). Solutions were prepared fresh, were protected from light exposure, and were added to the incubation medium in concentrations: 10 ng/ml, 100 ng/ml, and 250 ng/ml. The purity of the visfatin was in the range of 96% - 97% (SDS-PAGE analysis) and contained <0.01 ng µg⁻¹ LPS as determined by the Limulus amebocyte lysate method. HCT 116 cells were incubated with or without different concentration of visfatin 24 hours. During incubation period media from cells culture were not removed. After this time period cells were trypsinised (1% trypsin solution; PAA Laboratories) and collected by centrifugation (2000 rpm for 3 min).

**MTT assay**

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide salt (MTT, 5 mg/ml, Sigma-Aldrich, MA, USA). The cultures were incubated in 96-well plates at a density of 2 x 10⁴ cells per well. After 24 h incubation, cells were treated with various concentrations of visfatin/Nampt (10, 100 and 250 ng/ml) and cultured for 24 h. After incubation, 10 ml of MTT reagent was added to each well and incubated for 4 h at 37°C in the dark. The supernatant was aspirated and formazan crystals were dissolved in 200 ml of DMSO at 37°C for 15 min with gentle agitation. The absorbance was measured at 540 nm using the microplate reader model no. ELx800 (Bio Tec Ins). Data was normalized to the absorbance of wells containing media only (0%) and untreated cells (100%). Cell viability (%) was expressed as a percentage compared to the controls using the following equation: Cell viability [X] = Ns × 100/Nc; where Ns and Nc are the absorbance of wells containing media only (0%) and untreated cells (100%). Cell viability (%) was expressed as a percentage compared to the controls using the following equation: Cell viability [X] = Ns × 100/Nc; where Ns and Nc are the absorbance of surviving cells treated with sample solution and the absorbance of control, respectively. Each experiment was repeated eight times for each sample.

**Flow cytometry apoptosis/necrosis quantification**

Cells were seeded at a density of 1 x 10⁵/well in 24-well plates for 24 h before treatment of visfatin/Nampt (10, 100, 250 ng/ml) for 24 hours. Apoptosis was quantified 24 h after visfatin/Nampt treatment using a two-parameter fluorescence-activated cell sorting (FACS) analysis with annexin V/propidium iodide detection kit, according to manufacturer's instructions (In vitrogen). Briefly, cells were detached with trypsin (1%), washed with 1 x PBS and then resuspended in 1 x binding buffer (10 mM HEPES; 140 mM NaCl; 2.5 mM CaCl₂; pH 7.4), at a concentration of 1 x 10⁵ cells/ml. Afterwards, samples were...
stained with 5 mL annexin V conjugated with fluorescein isothiocyanate (FITC) and 5 mL propidium iodide (100 ng/ml); (PL), at room temperature for 15 min in the dark. They were then diluted in 400 mL of binding buffer (samples on ice) and analyzed within 1 h using a flow cytometer Aria III, (BD Biosciences); with parameter setting for FITC-Annexin V conjugates (488 nm laser line, LP mirror 503, BP filter 530/30), and for PI (488 nm laser line, LP mirror 566, BP filter 585/42). Four experiments repeated in triplicate were performed in this assay (n = 12).

Detection of reactive oxygen species using fluorimetric method with dichlorofluorescein

To monitor the intracellular ROS, we utilized cell-permeable oxidation sensitive fluorescent probes 5,6-carboxy-2′,7′-dichlorofluoresceindiacetate (DCFH-DA); (Molecular Probes, Leiden, Netherlands) using a fluorescent measurement system (Model Astroscan Cytofluor 2300/2350, Applied Biosystems, Millipore, Billerica, MA) as previously described. Non-fluorescent DCFH-DA, hydrolyzed to DCFH inside of cells, yields highly fluorescent DCF in the presence of intracellular hydrogen peroxide (H₂O₂). Therefore, the dichlorofluorescein (DCF) fluorescence intensity is proportional to the amount of intracellular ROS. Samples of 2 × 10⁵ cells were placed on Corning 6-well plates (Sigma-Aldrich, MA, USA) and were pre-incubated with 5 mM H₂DCF-DA for 1 h at 37°C. The plates were centrifuged at 1200 rpm for 10 min and the fluorescence of control and treated cells was read in the Cytofluor reader (excitation at 504 nm, emission at 526 nm). The background of deacetylated, oxidized 2′,7′-dichlorofluorescein (DCF) was approximately 60 – 75 relative fluorescent units (RFU). Each experiment was repeated eight times for each sample.

Detection of reactive oxygen species using flow cytometry and CellROX® Green reagent

Samples of 5 × 10⁵ cells were placed on Corning 6-well plates (Sigma-Aldrich, MA, USA) and were incubated with visfatin/eNampt (10, 100, 250 ng/ml) for 24 h in complete growth medium. After this period, CellROX Green Reagent (Molecular Probes, Poland) mixed with DMSO (Molecular Probes, Poland) was added to cells to a final concentration of 5 µM and incubated at 37°C in a 5% CO₂ humidified incubator for 30 min. Then cells were harvested, washed in PBS and analyzed on an Aria III flow cytometer (BD Biosciences). Fluorescence was read in the flow cytometry; on the FITC configuration (488 nm laser line, LP mirror 503, BP filter 530/30). Each experiment was repeated eight times for each sample.

Enzymes activity assays

Antioxidant enzyme activities: GSH-Px, CAT, and the level of malondialdehyde (MDA) were measured in cell supernatants. Cells were collected after 24 h of incubation with different doses of visfatin/eNampt (10, 100 and 250 ng/ml) and were centrifuged (2000 rpm, 5 min) and supernatants were kept at –80°C until analysis.

Cell lysates preparation

Control and visfatin/eNampt-treated HCT 116 cells were washed twice in ice-cold PBS and lysed at 4°C in 200 ml of lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris- HCl, 1% Triton X-100; all chemicals obtained from POCH Glwice, Poland) and then sonicated for 10 s. Cell lysates were obtained by centrifugation at 17,000 × g for 30 min at 4°C; protein concentration in the supernatant was determined by Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA, USA), and lysates were adjusted to equivalent concentrations with lysis buffer. Aliquots of 60 mg of total cell lysate were then frozen (–20°C) until antioxidant enzyme assays were performed.

GSH-Px activity assay

The method of Paglia and Valentine (34) was used with minor modifications as described previously (27) to measured GSH-Px activity in HCT 116 cell lysates. Firstly, human colorectal HCT 116 cells were pooled to a concentration of 5 × 10⁵ cells/ml. After centrifugation, the cell pellet was mixed with cell lysis buffer and then sonicated for 10 s (as described above). The protein concentration was measured using Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal volumes of each sample, containing 30 µg of protein were mixed with 2.68 ml of 0.05 M phosphate buffer (pH 7.0), containing 0.005 M ethylenediaminetetraacetic acid (EDTA). The following solutions were then added sequentially: 0.010 ml of glutathione reductase (GR), 0.100 ml of 0.0084 M NADPH, 0.01 ml of 1.125 M sodium nitrate (NaNO₃), and 0.1 ml of 0.15 M reduced glutathione (GSH). The enzymatic reaction was initiated by the addition of 0.1 ml of 0.0022 M H₂O₂. The conversion of NADPH to oxidised NADP⁺ was followed by continuous recording of the change in absorbency at 340 nm between 2 min and 4 min after the initiation of the reaction. The control measurements were recorded in a simultaneous assay where the sample was replaced by an equal volume of cell lysis buffer. 1 IU of GSH-Px enzyme activity is defined as 1 nM NADPH converted to NADP⁺ per mg of protein (IU/mg p). Analysis was carried out in duplicate repeated six times; (n = 12).

Catalase activity

Catalase activity was measured spectrophotometrically as described by Aebi et al. (35). Direct disappearance of 10 mM hydrogen peroxide in 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA was measured at 240 nm over 30 s on a Beckman DU-70 spectrophotometer. Enzyme activity was calculated based on the molar extinction coefficient of hydrogen peroxide at 240 nm (ε = 39.4 M⁻¹ cm⁻¹) and reported as µmol hydrogen peroxide decomposed per minute, recalculated to kIU per mg of protein (kIU/mg p.). Analysis was carried out in duplicate. Sample size in all experiments was 12.

Malondialdehyde assay

The measurement of MDA, a product of lipid peroxidation, was determined by the thiobarbituric acid (TBA) reaction as described by Placer et al. (36) and Ohkawa et al. (37) with minor modifications. Aliquots of reaction buffer (1.5 ml) that included 50 µg of protein from each sample and 1.4 ml of 0.2 M Tris - 0.16 M KCl (pH 7.4) were incubated at 37°C for 30 min. Next, 1.5 ml of TBA reagent were added, and the mixture was heated in a boiling water bath for 10 min. After cooling with ice, 3.0 ml of pyridine:n-butanol (3:1, v/v) and 1.0 ml of 1 M NaOH were added and mixed by shaking, and the absorbance was read at 548 nm. The blank control contained the same reaction mixture but was not incubated. The level of MDA was expressed as µmol MDA per mg of protein (µmol MDA/mg p.). Analysis was carried out in duplicate. Sample size in all experiments was 12 (four experiments repeated in triplicate).

Statistical analysis

Results are expressed as the mean ± standard deviation (S.D.). The normality of distribution was checked by means of
Shapiro-Wilk’s test. The statistical analysis of the data was performed using one-way ANOVA followed by the post hoc Tukey honestly significant difference test or Kruskal-Wallis test with Mann-Whitney tests according to variables distribution. The Bonferroni adjustment was applied for multiple comparisons. Differences were considered significant for P < 0.05. Statistical analysis was performed using a Statistica 7.0 software (Statsoft, Poland). According to experiments, sample size was 8 or 12; four experiments were performed in duplicate (n = 8) or in triplicate (n = 12), respectively.

RESULTS

The evaluation of the viability of human colon carcinoma cells HCT-116 using MTT assay

According to MTT assay, a decrease in viability of human colorectal HCT 116 cancer cells line was observed after treatment with visfatin/eNampt for 24 hours. The percentage of live cells after the treatment with visfatin/eNampt (10, 100, 250 ng/ml) was 89.2%, 87.7% and 83.8% compared to 97% in control cells; respectively (Fig. 1). The statistical significance was observed in all study groups when compared to control (untreated) cells.

The detection of reactive oxygen species using fluorimetric method with dichlorofluorescein

We tried to verify the hypothesis that occurring decrease in viability as well as increases apoptosis of HCT-116 cells treated with visfatin/eNampt was caused by changing the level of ROS following visfatin/eNampt treatment.

Mean fluorescence of untreated HCT-116 cells after 24 hours of culture resulted in 169.23 ± 5.3 RFU (relative fluorescence units). Visfatin/eNampt at 10 and 100 ng/ml reduced the ROS level 125.25 ± 10.72 RFU and 154.57 ± 5.47 RFU, respectively, compared to control cells 169.23 ± 5.3 RFU (both P < 0.05). On the other hand, visfatin/eNampt at 250 ng/ml resulted in a significant, 18% increase in ROS level after 24 h culture (199.57 ± 5.47 RFU vs. 169.23 ± 5.30 RFU; (P < 0.05)) (Fig. 3).

The detection of reactive oxygen species using flow cytometry and CellROX® Green reagent

Compared to control cells (771.3 ± 5.6 RFU), visfatin/eNampt at 10 and 100 ng/ml for 24 hours reduced ROS level (725 ± 9.6 RFU and 671.4 ± 1.5 RFU, respectively; both P < 0.05 vs. control cells). ROS level after visfatin/eNampt at 250 ng/ml remained unaffected compared to control cells (Fig. 4).

Activities of selected antioxidant enzymes and malondialdehyde concentration in cell lysates derived from cultures of HCT-116 cells

We tried to verify the hypothesis that ROS level in HCT-116 cells was dependent on antioxidant capacity of tested cells exposed to visfatin/eNampt, therefore we analyzed activity of selected antioxidant enzymes (CAT, GSH-Px) in HCT-116 cells exposed to visfatin/eNampt after 24 hours.

Catalase activity

Visfatin/eNampt at 10 and 100 ng/ml increased the catalase activity in HCT-116 cells compared to control cells (28.67 ± 3.53 kIU/mg and 25.18 ± 3.89 kIU/mg, respectively vs. 18.22 ± 1.05 kIU/mg in control cells; both P < 0.05). Interestingly, visfatin/eNampt at 250 ng/ml diminished the activity of catalase (14.14 ± 0.36 kIU/mg vs. 18.22 ± 1.05 kIU/mg; P < 0.05). (Fig. 5A).

Fig. 1. Viability of human colorectal HCT-116 cells line after visfatin/eNampt treatment for 24 hours. Cells were treated with various concentrations of visfatin/eNampt (10, 100 and 250 ng/ml) for 24 hours. Cell viability was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. Results represent the mean ± S.D. of four independent experiments repeated in duplicate (n = 8); *P < 0.05 vs. control.
Fig. 2. A representative FITC-staining flow cytometry analysis histogram for the visfatin-treated HCT-116 cells. For each FITC report, the lower left quadrant represents the detection of living cells, the lower right quadrant represents early apoptotic cells, the upper right quadrant represents late apoptosis cells, and the upper left quadrant represents necrotic cells after 24 h incubation with or without visfatin/eNampt (10, 100, 250 ng/ml).

Fig. 3. (A) Effects of visfatin/eNampt treatment (10, 100 and 250 ng/ml) for 24 hours on intracellular ROS level in HCT-116 cells using DCF-loaded cells (A). DCF-detectable ROS were measured in all study groups and control (untreated cells). Values were expressed as a relative fluorescence units (R.F.U) and were analyzed with one-way ANOVA test and post-hoc Bonferroni correction. Data are expressed as means ± S.D. (n.=12); a) $P < 0.05$ vs. control, b) $P < 0.05$ vs. visfatin (10 ng/ml)-treated group, c) $P < 0.05$ vs. visfatin (100 ng/ml)-treated group, d) $P < 0.05$ vs. visfatin (250 ng/ml)-treated group. (B) A representative merged images recorded by a confocal laser scanning microscopy (FluoView™ FV1000, Olympus, Japan), from ROS signals stained with DCF (FITC channel, green) and nucleus stained with DAPI (DAPI channel, blue), magnification × 40.
Glutathione peroxidase activity (GSH-Px)

Visfatin/eNampt at 10 ng/ml did not affect GSH-Px activity, but the increased concentration of visfatin/eNampt reaching 100 ng/ml resulted in an increase in GSH-Px activity compared to control cells (270.03 ± 8.72 IU/mg vs. 224.07 ± 4.89 IU/mg; P < 0.05). Nevertheless further increase in visfatin/eNampt concentration (250 ng/ml) caused a reduction in GSH-Px activity (197.02 ± 6.43 IU/mg vs. 224.07 ± 4.89 IU/mg; P < 0.05) (Fig. 5B).

The concentration of malondialdehyde

After 24 h of the treatment of HCT-116 cells with visfatin/eNampt at 10 and 100 ng/ml significant reductions in MDA levels were observed (1.42 ± 0.1 µmol MDA/mg and 1.01 ± 0.1 µmol MDA/mg vs. 1.76 ± 0.1 µmol MDA/mg in control group; P < 0.05). Contrary to the lower concentrations, visfatin/eNampt at 250 ng/ml increased MDA level in HCT-116 after 24 h treatment compared to control cells (2.36 ± 0.3 µmol MDA/mg vs. 1.76 ± 0.1 µmol MDA/mg; P < 0.05) (Fig. 5C).

DISCUSSION

In our current paper we showed a decrease of viability of human colorectal HCT 116 cells after visfatin/eNampt treatment. In MTT a linear increase in the percentage of dead cells (10.6%, 12.2% and 16.1%) was associated with increased concentrations of visfatin/eNampt (10, 100 and 250 ng/ml, respectively). In controls the percentage of dead cells reached 3%. Similar results were obtained using flow cytometry (6.6%, 9.8% and 16.3%, respectively). Cells were dying due to increased apoptosis rate (6.4%, 9.7%, 16.0%). Similar level of necrosis was seen in all studied groups and control cells. Recently, Jacques et al., showed that visfatin/eNampt together with 1 mM APO866 treatment (inhibitor of iNampt enzyme activity) induced apoptosis in 4.5% of murine articular chondrocytes, whereas visfatin/eNampt added solely to cell culture also induced apoptosis.
apoptosis in 0.5% of chondrocytes in vitro via unknown mechanism (28). The effects of visfatin/eNampt on viability/proliferation are dependent on cellular model used in study. Patel et al. showed visfatin-induced increases in cell proliferation in androgen-insensitive PC3 cells but not in androgen-sensitive LNCaP cells, suggesting that circulating visfatin can exert differing effects based on cell characteristics. They also showed that in LNCaP cells visfatin/eNampt protein was detected more abundantly in nucleus than cytosol when compared to PC-3 cells (33). Our previous findings also marked an influence of subcellular distribution of intracellular visfatin/eNampt (nucleus vs. cytosol) on proliferative status of HCT-116 cells cultured in vitro (38). It should be kept in mind that others authors have reported that visfatin/eNampt expresses anti-apoptotic effects in HUVECs (32), neutrophils (39), hepatocytes (40) and beta pancreatic islets cells (41). Cheng et al. showed that visfatin/eNampt reduces the percentage of apoptotic beta pancreatic cells induced by palmitic acid. This observation was associated by increased expression of Bcl-2 and reduced expression of caspase-3. PI3K/Akt and Erk1/2 kinases were responsible for the intracellular signaling in the above mentioned experiments (41).

We tried to verify the hypothesis that visfatin-induced decrease of viability of HCT-116 cells was dependent on changing ROS level followed visfatin treatment of tested cells. Reactive oxygen species (ROS) are side products of oxidative metabolism in living cells. They play important role in cell physiology, e.g. in intracellular signal transduction, proliferation, cell cycle control, cellular differentiation and aging (42-44). ROS at low concentrations tend to promote cell differentiation, whereas high concentrations induce cell death (apoptosis or necrosis) (45). Currently ROS are assayed in cell supernatants using fluorescence (fluorescence microscopy, flow cytometry, fluorometry) or confocal microscopy when a spacial distribution of ROS in cells is evaluated (46). In our paper quantitative analysis of ROS in human colorectal cells HCT-116 treated with various concentrations of visfatin was performed. Two different methods were employed in the study: (i) fluorometric analysis of the reaction between ROS and a H$_2$DCFDA producing a specific dye and (ii) flow cytometry with specific reagent for the detection of ROS - CellROX® Green reagent.

We showed that visfatin/eNampt at 10 and 100 ng/ml led to a decrease in ROS concentration in HCT-116 cells. Fluorescent method showed 26% and 23% in respective concentrations, whereas flow cytometry showed 6% and 13% reduction. On the other hand, visfatin/eNampt at 250 ng/ml was associated with a significant rise in ROS concentration (18%) that was detected in fluorescent method but it was absent in flow cytometry. Quantitative measurement of ROS in cells requires sophisticated research techniques. This is a consequence of short half-lives of ROS and activity of enzymatic and non-enzymatic pathways degrading ROS in living cells. DCF method is predominantly specific for the detection of hydrogen peroxide (H$_2$O$_2$). This is a result of a propensity of hydrogen peroxide to oxidize H$_2$DCFDA, which leads to the generation of fluorescent dye DCF. Hydroxyl radicals (OH•) do not participate in the transformation of H$_2$DCFDA into DCF. Therefore results derived from DCF-based methods of assessment of oxidative stress should be taken with caution. On the other hand Le Bel et al. stated that H$_2$DCFDA may be transformed into DCF by other oxide-reductive reactions and concluded that DCF method of ROS detection detects also other than H$_2$O$_2$, ROS in cells (47).

A positive association between oxidative damage (lipid peroxidation, DNA damage) and the number of apoptotic cells is seen. The accumulation of DNA and lipid damage lead to programmed cell death - apoptosis. Cai et al. (48) noted increased susceptibility to irradiation in colon cancer cell lines (LS1747 and HT-29) after stimulation with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). LS1747T colon cancer cell line is much more susceptible to ionizing radiation than HT-29 cell line. Addition of EPA and DHA increased apoptosis rate after irradiation of cell cultures, which may be a result of increased ROS generation, lipid peroxidation and DNA damage (48). In our current study we have shown that visfatin/eNampt administration to HCT-116 cell cultures resulted in a decrease of viability of these cells after 24 hours. However this effect was not linearly associated with ROS level. Visfatin/eNampt at 10 ng/ml even reduced ROS generation, but still a significant rise in apoptosis rate was observed. On the other hand high visfatin/eNampt concentration (250 ng/ml) resulted in the highest apoptosis rate (16% vs. 3.2% in control cells) which was accompanied by a substantial rise in ROS level (18% using DCF method) and MDA level (33%). These phenomena may stem from insufficiency in antioxidative pathways of HCT-116 cells when they are challenged with high concentration of visfatin/eNampt (250 ng/ml). At lower concentrations of visfatin the oxidative burst was blunted by the increased antioxidant enzymes (CAT - glutathione peroxidase) (41). ROS or RNS (reactive nitrogen species) might indeed be responsible for the additional oxidative damage that is not caused by hydrogen peroxide only, which finally may lead to increased apoptosis (50).

There are few studies exploring the in vitro generation of ROS in cells stimulated with visfatin/eNampt. Other researchers showed elevated levels of ROS after the administration of visfatin in culture medium in vascular smooth muscle cells (VSMC) (26), murine myocytes (C2C12) (24), and human melanoma cells (Me45) (25). The level of ROS was increased 2.5-fold in C2C12 cells treated with visfatin/eNampt (100 ng/ml) for 18 h and 3-fold in Me45 cells treated with visfatin/eNampt at the same concentration for 24 h; respectively (24, 25). Contrary to the above-mentioned studies we showed a modest (13%) decrease in ROS in similar culture conditions in human colorectal HCT-116 cells line. The increase in ROS was seen only when visfatin/eNampt at 250 ng/ml was used and was detectable solely in DCF method. This observation was not confirmed in flow cytometry using CellROX® green reagent, because it gave stronger fluorescence after intracellular macromolecule binding (DNA-specific dye), and then enhances oxidized signal from ROS - the first signal comes only from nucleus and mitochondria (51). Moreover, it is not excluded that other ROS scavenging enzymes such as: SOD isoenzymes and TRX enzyme may alters ROS level detected by CellROX in HCT-116 cells exposed to visfatin/eNampt.

These findings were followed by changes in MDA level after visfatin/eNampt treatment. In order to assess the changes in ROS and MDA level we evaluated the activity of selected antioxidant enzymes activity such as: CAT and GSH-Px. The diverse influence on H$_2$O$_2$ level in HCT-116 cells may result from the influence of visfatin/eNampt on antioxidant enzymes (catalase and glutathione peroxidase) that degrade hydrogen peroxide. Catalase (CAT) changes hydrogen peroxide into water and oxygen: 2H$_2$O$_2$ → 2H$_2$O + O$_2$. Glutathione peroxidase (GSH-Px) deals not only with hydrogen peroxide but also with lipid peroxides (52). Decreased concentrations of ROS may be attributable to the increased activity of CAT and GSH-Px during cell culture with 10 and 100 ng/ml of visfatin/eNampt. For
instance culture with visfatin/enampt for 24 hours resulted in 38% increase in CAT activity and 20% increase in GSH-Px activity, which was associated with reduced ROS concentrations. Interestingly in cells treated with visfatin/enampt at 250 ng/ml a significant rise in ROS was connected with the decrease in CAT and GSH-Px activities. Our previous study showed increased proliferation rate in melanoma Me45 cells that were treated with visfatin/enampt. We speculated that the influence of visfatin/enampt on cell proliferation depends on sufficient antioxidative response, which deals with ROS excess. Visfatin/enampt (100 and 250 ng/ml) greatly increased the CAT activity compared to control cells (32.1 kIU/mg p and 35.66 kIU/mg p vs. 7.55 kIU/mg p in control cells), which might reflect decrease in ROS level (27). In our current study on HCT-116 cells visfatin/enampt at 100 ng/ml slightly increased the activity of CAT (25.18 kIU/mg p vs. 18.22 kIU/mg p; P < 0.05) but at higher concentration (250 ng/ml) induced a drop in CAT activity (14.14 kIU/mg p vs. 18.22 kIU/mg p; P < 0.05).

The difference in the effects of visfatin/enampt on Me45 and HCT-116 cells may be a result of different proliferative/apoptotic reaction. Me45 cells showed increased proliferation rate after visfatin/enampt, while HCT-116 cells showed cytotoxic features and increased apoptosis. The final effect is therefore depending on ROS generation rate and the activity of antioxidant enzymes.

GSH-Px was similarly affected by visfatin/enampt in both Me45 and HCT-115 cells. Visfatin/enampt at 100 ng/ml doubled the GSH-Px activity in Me45 (210% of control) and significantly rised it in HCT-116 (120% of control). However when a visfatin/enampt concentration was increased to 250 ng/ml then GSH-Px activity was lowered in both Me45 and HCT-116 (60% and 80% of control, respectively). GSH-Px reduces not only hydrogen peroxide but also lipid peroxides. In HCT-116 cells visfatin/enampt at 100 ng/ml was associated with a reduced lipid peroxides assessed by MDA level, but 250 ng/ml of visfatin/enampt resulted in a 33% increase in MDA concentration. Buldak et al. (53) made similar observations in Me45 cells treated with visfatin/enampt. Difference in MDA level may reflect divergent intensity of oxidative-reductive processes in cultured control cells, which may affect further actions of visfatin/enampt on cells.

In summary, we showed that visfatin/enampt induces decrease of cell viability and apoptosis in human colon cancer HCT-116 cells. Visfatin/enampt affected the level of ROS level, however the association of ROS level and apoptosis rate was not linear. Moreover, elevation of antioxidant enzyme activities probably explains the drug resistance phenotype of some cancers cells (53), in this case, HCT-116 cells may cannot adaptive enough to ROS stress and apoptosis was occurs in cell culture.

Our study has a few limitations. Its in vitro setting may not fully reflect more complex relationships in organisms as a whole. The in vitro setting of the study must be kept in mind. The result may not be identical to those observed in living subjects. However authors put much effort on the exploration of oxidative stress, including two distinct methods of ROS assays and a broad spectrum of the experiments on activities of antioxidant enzymes.

Visfatin induced cytotoxic effects and promoted apoptosis in human colon cancer cells HCT-116 in vitro. Visfatin at lower concentrations (10, 100 ng/ml) reduced oxidative stress, but at high concentrations (250 ng/ml) it showed pro-oxidative features in HCT-116 cells. Visfatin at 100 ng/ml induced the activity of selected antioxidant enzymes (CAT, GSH-Px) in HCT-116 cell in vitro. However at the concentration of 250 ng/ml it reduced antioxidant cellular potential.

Acknowledgements: Biological experiments were performed in the Biotechnology Center of the Silesian University of Technology and Medical University of Silesia in Katowice using equipment financed by the “Silesian Biofarma” program; M.S. was also supported by grant No SUT-BK 256/Rau/1/2014 t.3 from Polish Ministry of Science.

Conflict of interests: None declared.

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Received: October 13, 2014
Accepted: April 22, 2015

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