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Response of OVCAR-3 Cells to Cisplatin and Hyperthermia: Does Hyperthermia Really Matter?

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Abstract. *Background: Hyperthermic intraperitoneal chemotherapy (HIPEC) is proposed as a promising treatment method, but fundamental information about the contribution of hyperthermia to intraperitoneal chemotherapy is lacking. The purpose of this study was to investigate the cytotoxic effect of hyperthermia and cisplatin on OVCAR-3 cells in vitro. Materials and Methods: Imitating the typical clinical conditions of HIPEC, OVCAR-3 cells were exposed to hyperthermia and cisplatin for 1 h. MTT viability test, flow cytometric analysis, and real-time cell and isobologram analysis were performed. Results: Hyperthermia up to 42˚C did not significantly increase the effect of cisplatin regarding the viability and apoptosis of OVCAR-3 cells. Moreover, an antagonistic effect of hyperthermia and cisplatin was revealed. Conclusion: Our investigation of OVCAR-3 cells critically disputes the benefit of hyperthermia in ovarian cancer treatment. Further in vitro and in vivo research is essential for better understanding of the mechanisms of action of hyperthermia and its role in the treatment of epithelial ovarian cancer.*

Ovarian cancer most frequently results in peritoneal carcinomatosis (PC). It is characterized by the presence of different sizes and numbers of tumour nodules on various sites of the peritoneal surface. PC confers a very poor prognosis (1). Unfortunately, advanced disease is often already present when the primary tumour is diagnosed (1).

The common treatment strategy for epithelial ovarian cancer (EOC) which has spread to the peritoneal cavity is a combination of cytoreductive surgery (CRS) whenever possible followed by systemic platinum–taxane-based chemotherapy (2). However, 20-30% of patients have a tumour which is resistant to systemic cisplatin from the onset, and nearly 70% of those who respond to platinum will experience relapse within 5 years (3). The introduction of intraperitoneal chemotherapy and regional hyperthermia (HIPEC) for the treatment of PC of gastrointestinal origin improved long-term survival in selected patients and also showed promising results in ovarian cancer (4). However, this advantage comes at the expense of increased toxicity and complication rates of up to 30%, as well as a reduced quality of life during treatment (5). In clinical trials, it was observed that spontaneous intestinal perforations can occur following HIPEC and may be related to the effect of localised heat and chemotherapy agents on traumatised bowel serosa (6). Moreover, recent investigations in rats showed that hyperthermia of 41.7˚C and higher was toxic to animals (7).

Generally, HIPEC is based on the rationale that hyperthermia affects cell membranes, the cytoskeleton, the synthesis of macromolecules, increases drug-induced DNA damage, inhibits the repair of drug-induced DNA damage (8), induces primary protein damage and influences the pharmacokinetics of drugs (9). Furthermore, mild hyperthermia has been shown to potentiate the antitumour effects of oxaliplatin, mitomycin C, and cisplatin (1). Based on its qualities, cisplatin is the most widely used chemotherapy agent for the treatment of PC (2). There are data about the synergistic effect of cisplatin and hyperthermia (42˚C) derived from malignant ascites (10) and an additive effect on the inhibition of cell–cell collaboration (11). On the contrary, some investigators have observed that heating cisplatin has no additional effect on cell viability and data about the effect of hyperthermia and cisplatin on ovarian cancer cells remain controversial (12, 13).

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Key Words: Epithelial ovarian cancer, HIPEC, cisplatin, hyperthermia, OVCAR-3.

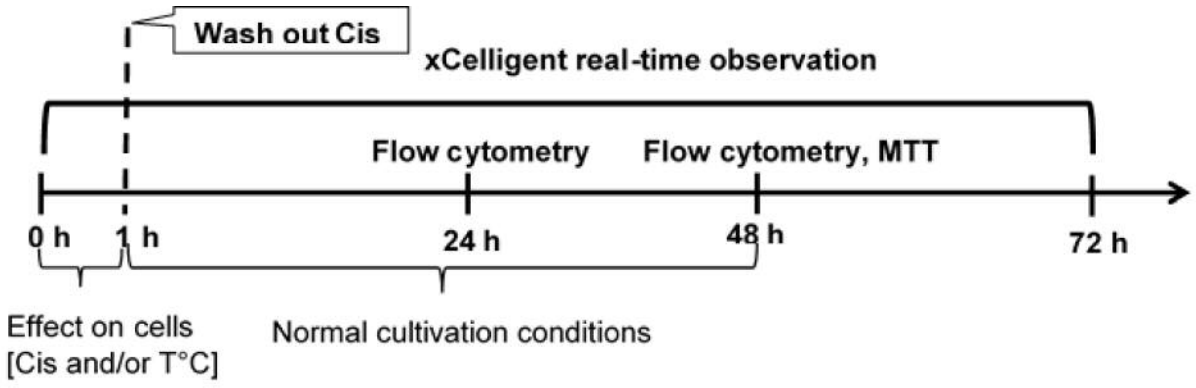

Figure 1. *Design of experiment. Cis: Cisplatin.*

Therefore, in this study, we analysed the response of OVCAR-3 cells to different hyperthermia and cisplatin combinations *in vitro*.

Materials and Methods

Cell line and culture conditions. Ovarian epithelial tumours account for the majority of all ovarian tumours; therefore, the OVCAR3 cell line was selected for the study. The human ovarian adenocarcinoma cell line NIH: OVCAR3 was obtained from the American Type Tissue Culture Collection (ATTC, Manassas, VA, USA). Cells were cultured in an incubator and maintained at a moist temperature of 37˚C, in a 5% CO2 enriched environment. Cells were grown in Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco/Invitrogen, Carlsbad, CA, USA) with 20% foetal bovine serum (FBS) (Gibco/Invitrogen), 1% penicillin/streptomycin solution (Gibco/Invitrogen) and 0.01 mg/ml bovine insulin (Sigma–Aldrich, St. Louis, MO, USA). Cells were grown under ATCC recommendations.

Design of experiment. OVCAR-3 cells were seeded in culture plates and cultivated for 24 h in the conditions described below. The effect of two single factors, temperature (37, 38, 39, 40, 41, 42, 43, 44 and 45˚C) and cisplatin (cis-diamminedichloroplatinum [II]) (0, 25, 50, 100, 200 and 400 μM) was investigated (for details see Figure 1). Imitating the typical clinical conditions of HIPEC, the cells were exposed to hyperthermia and cisplatin for 1 hour. Afterwards, the medium was washed out, and cells were returned to normal cultivation conditions (see description below) and incubated for 24 or 48 h. 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) viability test was performed after a 48-hour period. Flow cytometry for apoptosis analysis was undertaken after 24- and 48-h periods. The real-time effect of cisplatin and addition of temperature to cell viability was continuously monitored during all periods of the experiment by means of real-time cell analysis (RTCA) (Figure 1). All experiments were repeated at least three times.

MTT viability test. The MTT (Gibco/Invitrogen) assay was performed 48 h after the experiment. Cells were incubated with MTT (5 mg/ml) for 4 h at 37˚C. Following incubation the MTT solution was removed, and the formazan product was diluted by dimethyl sulphoxide (DMSO; Carl Roth GmbH, Karlsruhe, Germany), with gentle stirring for 5 min. The absorbance was then measured with a Sunrise spectrophotometer (Tecan GmbH, Grodig, Austria) at a wavelength of 570 nm and reference of 690 nm.

Table I. *Combination variables of cisplatin and temperature. Combination effect, reflected in cell viability in proc.*

Temperature (˚C)	Cisplatin concentration (μM)					
	0	25	50	100	200	400
37	100.0	102.2	95.4 2	52.5	21.2	18.6
38	97.9	92.9	78.7	52.1	17.5	11.4
39	97.4	99.3	77.7	43.2	15.8	11.8
40	95.4	102.1	76.6	30.3	9.8	8.2
41	91.4	90.9	68.3	25.9	9.5	8.4
42	92.7	99.3	74.2	32.8	12.3	10.5
43	89.2	75.9	42.9	19.0	10.4	9.7
44	84.0	59.2	28.0	16.2	11.9	9.6
45	79.3	46.5	21.0	14.2	10.1	5.8

Cell cytotoxicity analysis. RTCA was performed using the xCELLigence® RTCA DP real-time analyser (ACEA Biosciences, San Diego, CA, USA). It is based on the measurement of cell–sensor impedance in real time and gives the cell index a value that is directly influenced by the cell count. The cell index measurement was started immediately after seeding of the cells on an electronic microtitre plate (E-Plate). It was continued throughout the 24-h period until the experiment, during the 1-h experiment (with chosen combination of active factors: temperature of 37˚C with and without cisplatin, and a temperature of 43˚C with and without cisplatin) and continuing until the end of the experiment (Figure 1).

Isobolograms. To determine whether the cytotoxic effects of hyperthermia and cisplatin were antagonistic, additive, or synergistic, and to determine the confidence limits for the additive

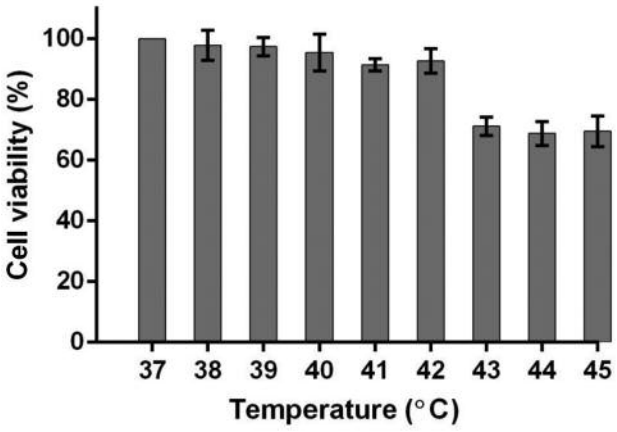

Figure 2. *The influence of temperature on OVCAR-3 cell viability. Following 1-h exposure of OVCAR-3 cells to different temperatures ranging from 37˚C to 42˚C, there was no significant change in cell viability. However, at temperatures of 43˚C and higher, the viability of cells significantly decreased by 21% ($p<0.05$). All data were compared with a control group (viability of cells at temperature of 37˚C set at 100%). Data are presented as the mean±standard error from ≥3 replicates.*

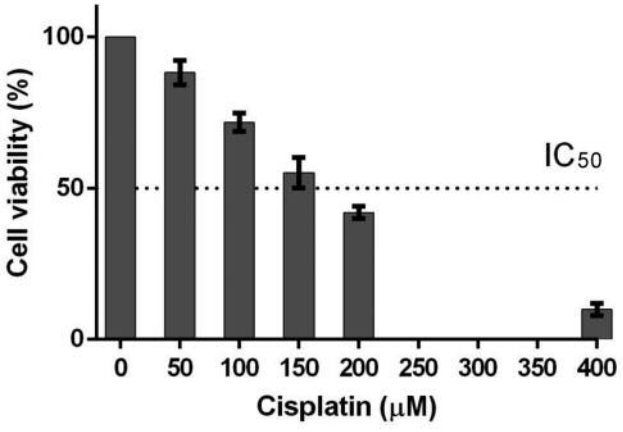

Figure 3. *The influence of different cisplatin doses on OVCAR-3 cell viability. One-hour exposure to gradually increasing doses of cisplatin had a negative influence on the viability of OVCAR-3 cells. The linear dependence between the dose of cisplatin and cell viability is evident. Half of the maximal inhibitory concentration of cisplatin for OVCAR-3 cells was found to be 152 μM. All data were compared with the control group (viability of cells at temperature of 37˚C set at 100%). Data are presented as the mean±standard error from ≥3 replicates.*

effect of two independent variables (temperature, cisplatin dose and a combination of both factors (which is marked in bold in Table I); isobologram analysis was performed according to Chou, Talarida, Steel and Peckham (14). The combination index (CI) was used to express synergism (CI<1), additivity (CI=1) or antagonism (CI>1) and was calculated according to the classic isobologram equation:

CI=d1/D1+d2/D2

where D1 and D2 represent the doses of cisplatin and temperature used alone to produce x% effect, and d1 and d2 are the doses of cisplatin and temperature used in combination to produce the same effect.

Analysis was performed using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

Cell apoptosis. Temperatures of 37˚C, 40˚C and 43˚C. were selected. Concomitant with exposure to different temperatures, OVCAR-3 cells were either exposed to half of the maximal inhibitory concentration of cisplatin (IC_{50}), or left untreated. The early apoptosis of cells was determined 24 and 48 h after the treatment. The procedure is based on the conjugation of phycoerythrin, annexin V and 7-amino-actinomycin D. All procedures were performed according to the manufacturer's instructions (Merck Millipore Billerica, MA USA). Apoptosis was evaluated by Guava Personal Cell Analysis Flow Cytometer (Merck, Millipore) using annexin V and phycoerythrin (FlowCellect™ MitoDamage Kit; Merck, Millipore).

Results

Limited effect of hyperthermia on cell viability. An increase of temperature from 37˚C to 42˚C had a very limited effect

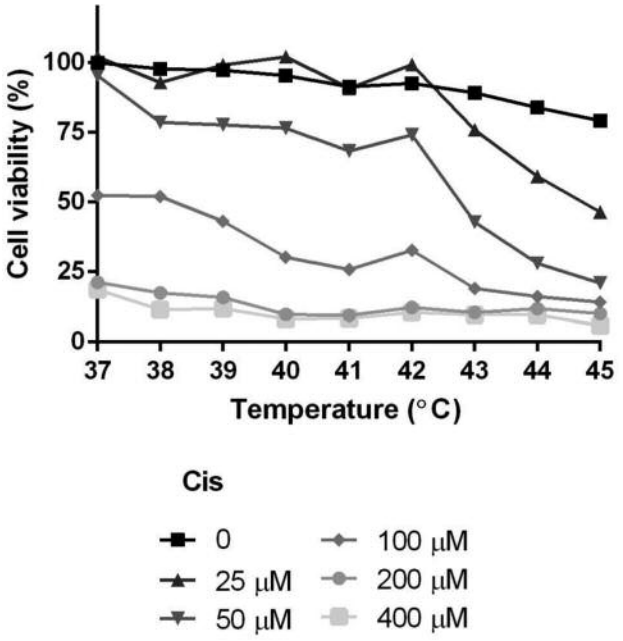

Figure 4. *The effect of different cisplatin doses and temperature combinations on OVCAR-3 cell viability. Hyperthermia up to 42˚C did not significantly enhance the effect of cisplatin on the viability of OVCAR-3 cells. However, there was a slight tendency for an additional effect of hyperthermia from 43˚C to 45˚C with intermediate doses of cisplatin (25, 50 and 100 μM). The viability of untreated cells in 37˚C was set at 100% as control. Data are presented as the mean±standard error from ≥3 replicates.*

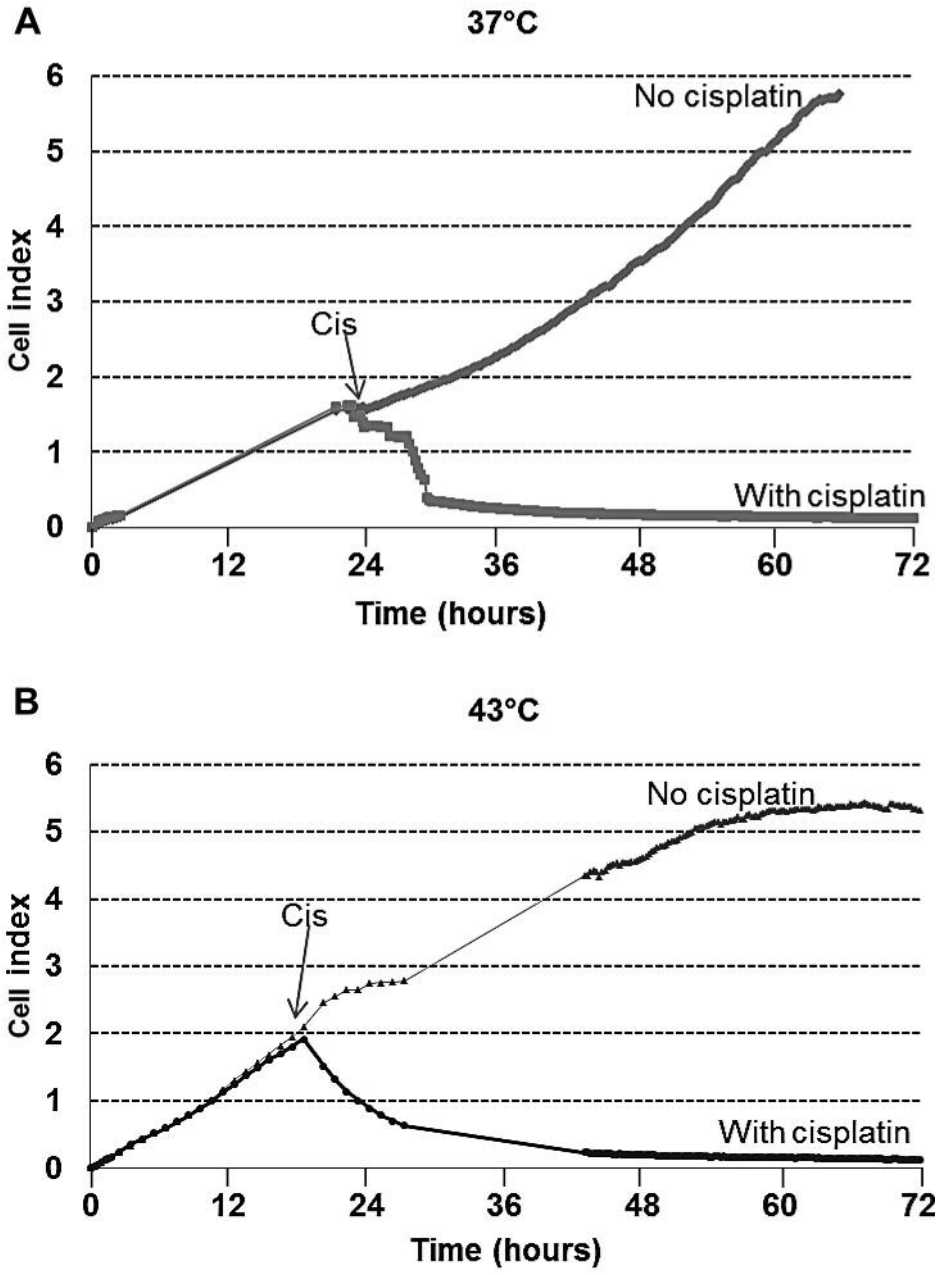

Figure 5. *Dynamic monitoring of OVCAR-3 cell viability using the xCELLigence system. The OVCAR-3 cells were incubated at 37˚C (A) and 43˚C (B). Viability of the cells exposed to 152 μM (half of the maximal inhibitory concentration of cisplatin) gradually decreased without significant differences under both experimental conditions (37˚C and 43˚C). Moreover, the viability of the untreated cells gradually increased under both temperatures. Data are presented as the mean±standard error from ≥3 replicates.*

on OVCAR-3 cell viability. At 43˚C, there was a marked decrease of cell viability (change of 21.51%). However, further increase of temperature had no further pronounced effect (Figure 2). We did not reach the half-maximal temperature; however, the quarter-maximal temperature (42.4˚C) was achieved.

Linear dependence of cell viability and dose of cisplatin. We examined the effect of cisplatin on cell viability. Increasing the dose of cisplatin reduced cell viability in a linear pattern. Consequently, the viability of OVCAR-3 cells was directly cisplatin dose-dependent. The IC_{50} was determined as 152 μM of cisplatin (Figure 3).

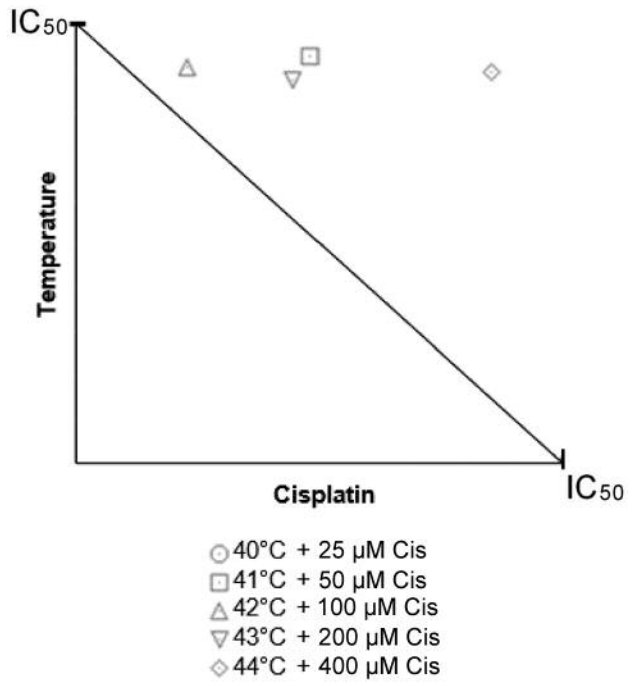

Figure 6. *Combined effect of cisplatin (Cis) and hyperthermia using isobologram analysis. The solid line in the diagram (combination index=1) indicates the alignment of theoretical values of an additive interaction between two effectors. Values above the solid line represent antagonistic interactions; values below the line represent synergism. With different combinations of cisplatin and hyperthermia, all data points are above the line, indicating a mathematically antagonistic effect of cisplatin and hyperthermia.*

The impact of cisplatin on cell viability is not potentiated by hyperthermia. An increase of temperature did not substantially improve the impact of different cisplatin doses on cell viability in our experiments (Figure 4): increasing temperature up to 42˚C had no impact on the effect of cisplatin. However, a meaningful additional effect was observed when the temperature was raised above 42˚C.

Continuous effect of cisplatin to viability of cells is not affected by hyperthermia. The results of RTCA show that the cell index increased exponentially at 37˚C without exposure to cisplatin. However, after 1-h exposure to cisplatin, the cell index decreased rapidly, until it reached a minimum (Figure 5A). The same pattern of cell index changes was observed at 43˚C. Thus, we observed no changes in cell growth pattern or in the effect of cisplatin, despite hyperthermia (Figure 5B).

Antagonistic effect of temperature and cisplatin. To investigate the potential synergistic anticancer effect of temperature and cisplatin, we treated OVCAR-3 cells using different combinations of these factors (Table I). The isobologram analysis revealed the antagonistic effects of hyperthermia and cisplatin combinations (CI>1) (Figure 6).

Apoptotic effect of temperature and cisplatin on OVCAR-3 cells. Flow cytometric analysis showed that exposure of OVCAR-3 cells to a temperature of 43˚C induced early apoptosis after 24 h in 16% of cells; however, this effect disappeared after 48 h (Figure 7A). At 37˚C, cisplatin (at IC50) induced early apoptosis in 18% of OVCAR-3 cells; the proportion of cells with signs of early apoptosis remained similar, even when the temperature was raised to 40˚C or 43˚C. In cisplatin-treated OVCAR-3 cells, the proportion of apoptotic cells remained stable after 24 and 48 h ($p<0.05$) (Figure 7B). There was no influence of hyperthermia on the proportion of apoptotic cells when exposed to cisplatin.

Discussion

Epithelial ovarian cancer is one of the malignancies that presents with PC, starting from stage II, according to the International Federation of Gynaecology and Obstetrics classification (15). Standard of care for the treatment of advanced ovarian cancer is CRS combined with adjuvant intravenous platinum–taxane chemotherapy (2, 16). Overall outcomes for patients with EOC remain relatively poor and after standard of care, the disease relapse is present in 60-70% of cases (3, 16). The peritoneal cavity is a common site of EOC dissemination (17) and recurrence (18), making it a good target for intraperitoneal chemotherapy (IPC), as systemic chemotherapy does not access non-vascularised cancer cells left in the peritoneal cavity during extensive surgery.

The rationale for intraperitoneal therapy is that the serous surface of the bowel and peritoneum is exposed to high concentrations of chemotherapy agents (5). IPC following CRS has a significant impact on overall and progression-free survival in advanced ovarian cancer (13). Sugarbaker et al. reported an improvement in the treatment of PC following the introduction of HIPEC (19).

HIPEC, combining regional hyperthermia and IPC after CRS, has been applied to treat PC and is the most effective and recommended treatment option for appendiceal mucinous cancer and *pseudomyxoma peritonei* (20). For a number of malignancies that present with PC, including peritoneal mesothelioma (21), and gastric (22), colorectal (23) and pancreatic (24) cancer, CRS and HIPEC prolonged long-term survival in selected patients. Some oncology centres, encouraged by the promising results of HIPEC in the treatment of PC of gastrointestinal origin, have shifted this method as a treatment option to patients with advanced EOC (25). Despite the lack of strong scientific evidence, *in vitro* and *in vivo* clinical trials to evaluate the role of HIPEC in EOC were implemented. An increased overall survival rate was detected in selected patients with ovarian cancer. (4).

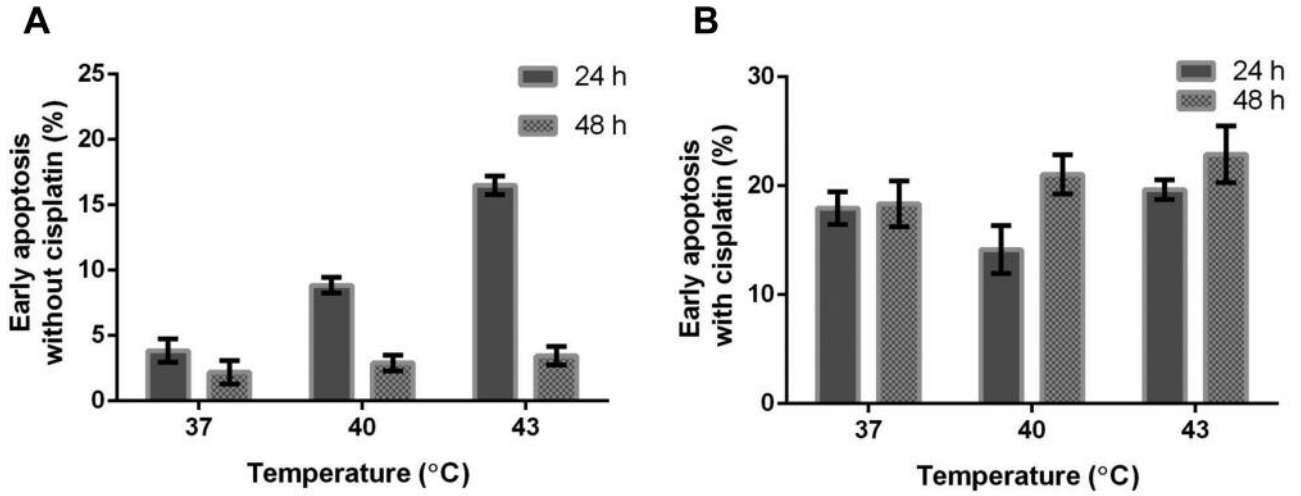

Figure 7. *Apoptotic effect of temperature and cisplatin on OVCAR-3 cells. OVCAR-3 cells were incubated at different temperatures (37˚C, 40˚C, 43˚C) for 1 h with and without exposure to half of the maximal inhibitory concentration of cisplatin. Cells were then incubated with phycoerythrin–annexin V in a buffer containing 7-amino-actinomycin D for 24 and 48 h following the experiment and cells were analysed by flow cytometry. Twenty-four hours following 1-h incubation, the proportion of cells with signs of early apoptosis increased to 9% at 40˚C and to 16% at 43˚C. However, this effect disappeared after 48 h (A). Cisplatin treatment resulted in an increase in the number of apoptotic cells at 37, 40, and 43˚C without a significant difference after 24 and 48 h following the experiment (B).*

However, complete remission was not attained (26). Five-year disease-free survival was only 6% in the HIPEC-treated group (27). Moreover, a recently published critical review of clinical trials of HIPEC in ovarian cancer did not find any benefits in overall and disease-free survival (28). Currently, the use of HIPEC in clinical practice for the treatment of ovarian cancer is under discussion.

Fundamental information regarding the contribution of hyperthermia to IPC in EOC is poor and controversial. Some authors hypothesised that heating the chemotherapeutic agent may eliminate any remaining cancer cells in the peritoneal cavity after extensive CRS. Little information is available on the interaction between cisplatin and hyperthermia at the cellular level in ovarian cancer.

We investigated the effect of cisplatin and hyperthermia *in vitro* on OVCAR-3 cell viability and apoptosis under conditions resembling those found during HIPEC (12). We analyzed the effect of 37˚C as a baseline to verify the effect of normothermia, and 40-43˚C, a commonly used temperature for HIPEC in clinical practice. The hyperthermia investigated in the most detail was 43˚C, while hyperthermia exceeding 44˚C, as used in previous studies (13, 29), is not rational and not conceivable in animal models or clinical practice. We have demonstrated that hyperthermia up to 42˚C had no effect on OVCAR-3 cell viability; moreover, as shown in real-time analysis, hyperthermia actually potentiated the growth of cancer cells.

Hyperthermia at 43˚C has a markedly negative effect on cell viability; however, we did not reach a level that killed 50% of the OVCAR-3 cells in our experiments. The exposure of cell lines to a temperature of 45˚C achieved such a killing effect and initiated the induction of heat-shock protein HSP60 in experiments by Kimura and Howell (13); however, in our experiments, the exposure of OVCAR-3 cells to 45˚C led to only a 25% negative effect on viability (data not shown). Experimental data on the effect of hyperthermia at temperatures exceeding 43˚C are not applicable *in vivo*, as seen for the HIPEC model discussed above. Moreover, recent investigations in rats showed that hyperthermia of 41.7˚C and higher was itself toxic to animals (7). Following exposure to temperatures above 44˚C, the mortality rate of rats exceeded 50% (30). Hyperthermia of 40.5 to 41.5˚C as a single factor increased the median survival of rats from 9 to 22.5 days, but a consequence was a postoperative complication rate of 14%, including mesenteric ischaemia, bowel necrosis and intra-abdominal bleeding (7). It was observed in clinical trials that spontaneous intestinal perforations can occur following HIPEC and may be related to the effect of localised heat and chemotherapy agents on traumatised bowel serosa (6).

We have demonstrated that the effect of cisplatin is substantially dose-dependent and there is a negative linear pattern of the dependency of OVCAR-3 cell viability on cisplatin dose. Hyperthermia up to 42˚C, here with cisplatin, had no additional effect on cell viability, in accordance with the data of other investigators (8). The impact of temperatures of 43˚C and higher was highlighted alongside lower cisplatin

doses. The treatment of OVCAR-3 cells with cisplatin led to the induction of early apoptosis. However, hyperthermia had no influence on the apoptotic effect of cisplatin. Moreover, isobologram analysis (14) revealed the antagonistic effect of hyperthermia and cisplatin in OVCAR-3 cells.

The interaction of cisplatin and hyperthermia *in vivo* is more complex and extensive than at the cellular level. The response of cancer cells *in vivo* is influenced by factors such as drug metabolism, tumour vasculature, immune response, drug-resistant cell subpopulations, and cellular and extracellular matrix interactions (31).

In conclusion, our investigation of OVCAR-3 cells critically disputes the benefit of hyperthermia in ovarian cancer treatment. Furthermore *in vitro* and *in vivo* research is essential for a better understanding of the mechanisms of action of hyperthermia and its role in the treatment of EOC.

Acknowledgements

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