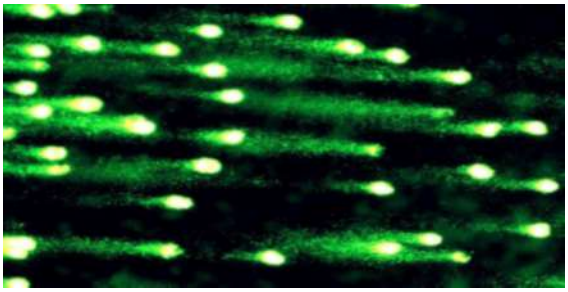
BUKU PROFIL PENYELIDIKAN SKIM GERAN PENYELIDIKAN FUNDAMENTAL (FRGS) FASA 1/2013 DAN FASA 2/2013



TARGETING HEAT SHOCK RESPONSE IN OSTEOSARCOMA CELLS BY COMBINATION TREATMENT

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ABSTRACT (120 words)

Metformin has anticancer effects by inhibiting glucose metabolism. Hyperthermia sensitizes cancer cells. It is known that both metformin,hypo- and hyperthermia deregulate mitochondrial metabolism in cancer. However, the mechanism of the additive effect is not known. The combined effect of metformin and hypo- or hyperthermia on osteosarcoma cell line (MG-63 cell) viability, DNA damage, glucose metabolism, regulation of apoptotic genes and proteins were investigated. Osteosarcoma (MG-63) cells were treated with metformin IC50 30M for 48h followed by exposure to 45°C, 39°C, 35°C 27°C for 30 min, 1h and 2h , 37°C was served as control. The combinations of metformin with hypo- and hyperthermia significantly downregulated the expression of AKT1 and GSK3(3 genes, which led to a reduce cells viability and an increase in DNA damage. Metformin induced apoptosis and necrosis. The combined effect caused a downregulation of anti-apoptotic Bcl-2. The intrinsic pathway was not activated. Therefore, this study suggests that the combination of metformin with hyperthermia particularly 45°C, enhances apoptosis via Bax/Bid-dependent pathway.

1. INTRODUCTION

Osteosarcoma is a malignant bone cancer associated with development of chemotherapy resistance. Hypo- and hyperthermia have been used as an adjuvant to radiation therapy as both seem to have complimentary effects. Therefore this study aims to combine temperature stress which causes cell cycle arrest and DNA damage with drugs that interfere with the energy metabolism and/or induce apoptosis. Metformin is known as antiglycemic drug with anticancer properties. Metformin has multiple potential applications. Metformin was associated with decreased cancer incidence and mortality in diabetic patients and the insulin-lowering effects of metformin may be integral to its anticancer properties. In cancer, metformin displays significant growth inhibitory effects in several cancer cells and mouse tumor models. Meanwhile, it has been suggested that metformin uncouples the TCA cycle from oxidative phosphorylation and therefore reduce energy production. In combination with hypo- and hyperthermia, it may increase the mitochondrial apoptotic pathway and prevent DNA damage repair program.

Osteosarcoma is a rare but very aggressive malignant bone cancer. Despite surgery and chemotherapy, osteosarcoma has a high recurrence rate and is associated with the development of chemotherapy resistance. Therefore the search for alternative treatment modalities is still on. Hypo- and hyperthermia have been used as adjuvant mainly to radiation and chemotherapy and it has shown to be beneficial and increasing survival rates in several types of cancers. However the treatment of osteosarcoma cells with hypo- or hyperthermia is not sufficient and an additional treatment is necessary. Metformin interfere with the cells energy production and therefore reduce the availability of ATP for other prosurvival pathways. Therefore it stands to rationale that metformin would be a good compliment treatment with hypo- and hyperthermia for osteosarcoma.

1. RESEARCH METHODOLOGY

Osteosarcoma cells (MG-63) were first cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Approximately 2,500 MG-63 cells/well were seeded into 96-well plates and tested for metformin IC50 dose. Once the concentration of the dose was established (IC5o=30M for 48h), MG-63 viability was measured using MTS assay. Cells were preincubated

with metformin for 48h and exposed to moderate (35°C) and severe (27°C) hypo- also to moderate (39°C) and severe (45°C) hyperthermia for 30mins, 1h and 2h. After each time points, damage to DNA was accessed by comet assay stained with SYBR® Green and type of cell death was determined via Annexin V-FITC and PI staining. Gene and protein expression of AKT1 & GSK3P (glucose metabolism), DR5, Bax, Bid, Bcl-2, AIF, cytochrome c, Apaf1, Caspase 8, 9 & 3 (apoptosis) were measured using real-time PCR and ELISA.

2. LITERATURE REVIEW

Metformin is the most widely used hypoglycemic drug for treatment of type 2 diabetes [1]. Accumulating evidences in recent years clearly showed that metformin possesses significant anti-cancer effects [1]. For instance, the incidences of various cancer and cancer-related mortality have been found to be significantly lower in type 2 diabetic patients treated with metformin than in those treated with other types of anti-diabetes drugs. Numerous pre-clinical studies have shown that metformin suppresses proliferation and induces apoptotic death in various cancer cells [2-3]. Metformin has also been shown to prevent lung tumorigenesis caused by tobacco carcinogens and enhance the response of experimental tumors to chemotherapy and radiotherapy.

A number of divergent cellular and molecular mechanisms have been proposed to account for the anti-cancer effects of metformin. The mechanism by which metformin produces its inhibitory effect on cancer development and tumor growth is not completely understood. These could be through indirect effects on systemic levels of insulin or glucose or through direct effects on tumor cell growth and survival. However it is known that metformin disrupts the oxidative phosphorylation in mitochondria, thereby decreasing ATP level and concomitantly increasing AMP level [1]. Direct effects of metformin on cancer cells include inhibition of cell proliferation and induction of cell death.

In the studies where metformin has been shown to promote cell death, the mechanism appears to involve activation of apoptotic pathways. Nontransformed breast epithelial cells were also resistant to the cytotoxic effects of metformin [3]. In sensitive cell lines, cell death was mediated by both caspase-dependent and caspase-independent mechanisms. The caspase-independent pathway involved activation of poly(ADP-ribose) polymerase (PARP), was associated with mitochondrial enlargement, and was reduced by depletion of apoptosis inducing factor (AIF) [2]. It is well-established that moderate hyperthermia at 39-43°C kills cancer cells and sensitizes cancer cells to chemotherapy or radiotherapy. Lee et al (2014) showed that metformin is preferentially cytotoxic to cancer stem cells (CSCs) relative to non- CSCs and that hyperthermia markedly increase the metformin cytotoxicity against CSCs [1].

3. FINDINGS

In this research a combination of Metformin and hypo- or hyperthermia was applied. However before that, the 50% (IC50) cell death for MG-63 cells was determined after 48h of incubation. We could show that 30M of Metformin was sufficient to kill 50% of MG-63 cells. Next, we determined the combined effect of Metformin and various temperatures on MG-63 cells after 30mins, 1h and 2h. Our results showed that increase in temperature severity and incubation time lead to significant reduction in cell viability. This was observed when MG-63 cells preincubated with Metformin followed by exposure to severe hyperthermia at 45°C resulted in significant reduction in cell viability. Next we examined if combined treatment of Metfomin and temperature leads to DNA damage. By accessing with Comet assay stained with SYBR® Green, MG-63 cells in combination with Metformin and severe hyperthermia conditioning lead to

Grade 4 DNA damage. Even though DNA damage was observed with response to moderate and severe hypothermia, damage was graded at stage 1, 2 and 3. Our results showed that increase in DNA damage responded according to temperature severity.

Given that DNA damage was observed with response to combined Metformin and hypo- or hyperthermia treatment, we measured the rate of apoptotic cells to determine if cells were undergoing apoptosis. The combined treatment of Metformin with hypo- or hyperthermia increased the rate of apoptosis compared to cells treated with Metformin alone. Significant increase in apoptosis was observed when MG-63 cells exposed to 45°C.

MG-63 cells that were treated with hypo- and hyperthermia in combination with metformin expressed higher levels of Hsp60 as compared to cells treated with metformin alone. This suggests the role of this protein in chaperoning temperature-induced protein misfolding. Meanwhile the expression of Hsp27 and Hsp70 was reduced in response to hypothermia and hyperthermia suggesting their anti-apoptotic function was halted in response to temperature exposure. The apoptosis inducing factor (AIF) was overexpressed in response to severe hyperthermia. Expression of intrinsic apoptotic caspase-9 was seen to be increased in response to both moderate and severe hypo- and hyperthermia at all time points. The execution of apoptosis measured by caspase-3 was expressed at basal level except in response to 2 h of severe hyperthermia. No combined effect was observed in response to moderate and severe hypo- and hyperthermia except at 2 h of severe hyperthermia.

The combination of metformin and hyperthermia significantly downregulated the expression of AKT1 and GSK3/3, which in turn led to a reduction in cell viability and an increase in DNA damage. The combined effect caused an upregulation of DR5, Bax, Bid found upstream of mitochondrial signalling molecules and a downregulation of anti-apoptotic Bcl-2. The intrinsic pathway was not activated as the expression of AIF and cytochrome c was downregulated. Although Apaf-1 upregulated caspase 9, caspase 3 remained downregulated.

4. CONCLUSION

By looking at the results we have up to date, damage to DNA and rate of apoptosis are highly dependent on temperature severity and exposure duration. The adjuvant effect of metformin with severe hyperthermia showed increase in DNA damage and apoptosis, suggesting a possible anticancer activity of metformin. The combination of metformin with hyperthermia particularly 45°C, enhances apoptosis via Bax/Bid-dependent pathway. This pathway is either directly p53-mediated or through direct activation of caspase 7.

ACHIEVEMENT

I. Name of articles/ manuscripts/ books published:

1- A Critical Link between Advanced Glycation Endproducts, Osteoporosis and Diabetes Mellitus (under review)(JKSUS), (Scopus index)

2- Combination Of Metformin And Severe Hyperthermia Induces DNA Damage and Apoptosis in Osteosarcoma Cells in Vitro (Abstract in Malaysian J Pathol 2015; 37(2) : 175 - 211) (IF=0.26)

3- Severe hyperthermia induces DNA damage, apoptosis, and suppression of heat shock protein 90a expression in osteosarcoma cells, (under review) (Oncology Reports. IF 2.486)

II. Title of Paper presentations (international/ local)

International:

1. Synergistic Effect of Metformin and Severe Hyperthermia on Osteosarcoma Cells. **(International Conference of Advances in Medical Sciences , ICAMs , Concorde Hotel KL 15-16 /April 2015)**

2. Combination of severe hypothermia with metformin enhances apoptosis in osteosarcoma cells. (**International Conference On Molecular Biology And Biotechnology (9-11th March 2016 Connexion@Nexus, Kuala Lumpur)**

Local:

1- Combination Treatment Reduces Osteosarcoma Cell Survival by Interfering with Heat Shock Response. **(Research week, 5th - 9th October, 2015 Faculty of Medicine Sungai Buloh Campus)**

2- Combination of Metformin and Severe Hyperthermia Activates Bax/Bid-Dependent Apoptosis in Osteosarcoma Cells in Vitro. **(2015 Annual Scientific Meeting College of Pathologists Academy of Medicine Malaysia and 40th anniversary Celebration of Pathology Advocates 13-14 June 2015 Berjaya Times Square Hotel KL)**

3- Berberine Increases Moderate But Not Severe Hyperthermia Induced Cell Death In Osteosarcoma Mg-63 Cells.**(2015 Annual Scientific Meeting College of Pathologists Academy of Medicine Malaysia and 40th anniversary Celebration of Pathology Advocates 13-14 June 2015 Berjaya Times Square Hotel KL)**

III. Human Capital Development:

1-Mohammed Ali Orba Nashiry 2010658578 PhD
2- Aisha Ninti Mohd Din Research Assistant

IV. Awards/ Others :

1st Prize Best poster Presentation. **(2015 Annual Scientific Meeting College of Pathologists Academy of Medicine Malaysia and 40th anniversary Celebration of Pathology Advocates 13-14 June 2015 Berjaya Times Square Hotel KL)**