

Metformin may offer no protective effect in men undergoing external beam radiation therapy for prostate cancer

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Running Title: Metformin offers no protection in Prostate Cancer

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/BJU.14709](https://doi.org/10.1111/BJU.14709)

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Article type : Original Article

Article Category: Translational Science

Abstract

Objectives:

Metformin, which inhibits mammalian target of rapamycin (mTOR), has demonstrated protective effects in prostate cancer (PC). It can reduce hypoxia inducible factor 1- α (HIF1 α), which normally confers radio-resistance when upregulated via the PI3K/mTOR pathway. Therefore, we aimed to assess if metformin reduced radio-resistance and increased survival in men undergoing external beam radiotherapy (EBRT) for PC, and to determine its effect on HIF1 α .

Subjects/patients (or materials) and methods:

All patients treated with curative intent with EBRT for PC at a major cancer centre from 2000-2007 were included. The outcome measures of time to biochemical failure (BF), metastasis, PC specific mortality and overall survival (OS) were analysed in those taking metformin vs those not, using competing risk and Cox regression models. To determine metformin's effect on HIF1 α expression and survival in vitro, PC3 cells with high basal HIF1 α levels were subjected to increasing doses of metformin after H₂O₂-induced oxidative stress.

Results:

A total of 2055 eligible cases, including 113 who were on metformin, were identified with a median follow-up of 95.7 months. There were no differences in age, initial PSA, Gleason scores, T stage, D'Amico risk or duration of ADT between men who were or were not on metformin. Treatment with metformin did not result in any apparent improvement in time to BF, time to metastasis detection or overall survival, but there was a 1.5-fold increase in PC-specific deaths ($p = 0.045$) in men on metformin and ADT when adjusted for cancer risk and co-morbidities. When comparing men on high dose metformin ($>1\text{gm/d}$) with low dose metformin ($\leq 1\text{g}$), there was no difference in either time to metastases or time to BF. In vitro metformin at a high concentration of $100\mu\text{M}$ did not reduce HIF1 α expression nor did metformin affect the PC3 cell survival when exposed to oxidative stress (H₂O₂).

Conclusions:

No association was found between the use of metformin and time to metastasis detection, time to BF or overall survival in men undergoing radiation therapy +/- ADT for PC. In vitro, low therapeutic concentrations of metformin had no effect on HIF1 α , and this observation could explain the conflicting evidence for the effectiveness of metformin in men undergoing EBRT for PC. Higher, more toxic doses of metformin may be required to inhibit the mTOR-HIF1 α pathway in this patient group.

Introduction

Prostate cancer (PC) is the second most common cancer in men with an estimated 1.1 million cases diagnosed worldwide in 2012 (1). External beam radiotherapy (EBRT) with androgen deprivation therapy (ADT) is one of the standard modalities of curative treatment for localised PC(2, 3).

Metformin is a biguanide anti-hyperglycaemic agent used in type 2 diabetes because of its significant benefits in reducing cardiovascular disease (4). Thus, the use of metformin in PC has received much recent attention because of its potential role in prostate cancer protection and especially in ameliorating the metabolic effects encountered during the use of (ADT) (4, 5). Furthermore, in several studies, metformin was shown to have protective effects in PC with reduced castrate resistance, early stabilisation of PSA in castrate resistant disease and improved overall survival (6-9). However, the evidence is conflicting (5, 10).

Metformin is thought to have anti-tumorigenic activity not only by direct action on the tumour but also indirectly by lowering systemic insulin levels. Both pathways activate AMPK, which in turn inhibits the mammalian target of rapamycin (mTOR) and causes reduced cell proliferation (11, 12). Hypoxia-inducible factor 1 α (HIF1 α) is a transcription factor which promotes cancer growth, cell proliferation, angiogenesis, metastases (13) and survival of prostate cancer cells (14). In addition, HIF1 α has been shown to increase resistance to radiotherapy (15), and targeting the mTOR/HIF1 α pathway improves radio sensitivity in endometrial cancer (16). As mTOR is an upstream regulator of HIF1 α (17), we

hypothesized that metformin should reverse the effects of HIF1 α , thereby reducing radio-resistance and increasing survival in men undergoing EBRT for PC, and increasing the sensitivity of PC cells to H₂O₂ toxicity. Therefore, we aimed to assess the effects of metformin on PC outcomes in men treated with EBRT and to determine its effect on HIF1 α and cell survival *in vitro*.

Subjects/patients (or materials) and methods

All patients, with histological evidence of adenocarcinoma of the prostate and clinical stage T1-3b Nx/0 M0, who were treated with curative intent with EBRT or combined EBRT+ High dose rate (HDR) boost at the Peter McCallum Cancer Center (PMCC) from 2000-2007 inclusive and who had a minimum potential follow-up of 5 years were included.

The following data were collected: pre-treatment prostate specific antigen (18) (at least one), Gleason score on biopsy, clinical T stage, family history of PC, previous cancer history, comorbidity information (Adult Comorbidity Evaluation (ACE)-27) and the use of metformin and relevant associated medications (aspirin, statins) as well as the duration of adjuvant ADT (short-term or long-term). ACE-27 is a validated co-morbidity index, which takes into account all systems including the cardiovascular system and is widely used in cancer studies including prostate cancer(19) and was used to assess and score cardiovascular and co-morbidities rather than cardiac disease individually. The following outcome measures of interest were analysed: all follow up PSA values, use of salvage androgen deprivation therapy, time to metastases and time to and cause of death using.

For all cases with data missing for >18 months, or aberrant data (such as a falling PSA in the absence of recorded therapy), the patient medical record was reviewed and where no data was available for a period of >2.5 years, the patient was deemed to be lost to follow-up at the start of that period.

Endpoints

The primary endpoint was based on biochemical failure (BF), which was defined by the Phoenix criteria (20), whereby failure is called at the date at which the PSA reaches a level of 2 ng/mL or more above the lowest PSA recorded previously. Time to BF was taken as the interval from completing radiotherapy to the date of BF. Secondary endpoints were time to commencing salvage ADT, time to distant relapse (metastasis), and time to death from PC or any cause.

In vitro studies

Cell culture

The human prostate cancer cell lines PC3 and LNCaP were cultured in RPMI 1640 medium (Invitrogen, Mulgrave, Australia) supplemented with 7.5% FBS and 100 U/mL penicillin and streptomycin. All cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO₂.

Western blot analysis

PC3 and LNCaP cells were incubated with the indicated concentration of metformin or ammonium ferric citrate in serum free medium (SFM) for 18-24 h. Western blot analysis of HIF1 α was performed as previously described (21) with a monoclonal mouse anti-human HIF1 α antibody (1:1000, BD Biosciences, North Ryde, Australia) followed by a secondary goat anti-mouse horseradish peroxidase-conjugated antibody (1:5000, Bio-Rad). As a loading control, blots were incubated with a horseradish peroxidase (HRP)-conjugated rabbit anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized in a LAS 3000 Image Reader (Fujifilm, Brookvale, Australia), with an ECL Advance Western Blotting Detection Kit (GE Healthcare).

Survival assay

PC3 and LNCaP cells were plated on a 12-well plate (1.2×10^5 cells/well) and incubated in culture medium for 24 h. Metformin in SFM was added to the indicated final concentrations and the cells were incubated for 4 h. H₂O₂ to a final concentration of either 50 μ M in case of PC3 and 25 μ M in case of LNCaP cells from a stock solution of 50mM or 25mM respectively

was added directly in the well and the cells were incubated for further 18 h. For Fe^{2+} , ammonium ferric citrate in SFM was added to the indicated final concentrations and the cells were incubated for 4 h, and the media was then replaced with 50 μM or 25 μM H_2O_2 diluted in SFM and incubated for a further 18 h. Following the incubation relative cell numbers were determined using a MTT assay. MTT stock solution (5mg/ml) (Sigma Aldrich, USA) was prepared in 1x PBS and 10 μL /well added. After incubation with MTT solution for 1-2 h and following the solubilization of formazan crystals in acidified isopropanol the absorbance, which is directly proportional to cell numbers, was measured at a wavelength of 570 nm with background subtraction at 620 nm using a FLUOstar Optima Microplate Reader (BMG Labtech, Mornington, VIC). Data are expressed as a percentage of the untreated controls.

Statistical Analysis

Clinical data

Given that metformin usage is of interest in terms of tumour control but may also impact on risk of death from other causes, we also modelled effects using a competing risks framework for all endpoints other than overall survival, which uses a Kaplan Meier model as by definition, no competing events are possible for survival. Differences in endpoints were expressed as hazard ratios or actuarial rates, with 95% confidence intervals. Descriptive data were displayed as median and 95th intercentile range (encompassing the 2.5th to 97.5th centile range) for continuous data, or as counts and proportions for categorical data. Differences in these types of data was assessed using Wilcoxon and Pearson chi-square tests respectively. All analyses were 2 sided with a $p \leq 0.05$ deemed significant and were performed using the R statistical language.

Pre-clinical data

Data are presented as means \pm SEM. Statistical significance for single comparisons of normally distributed data was determined by Student's t test or for data that was not normally distributed by Mann-Whitney rank sum test. For multiple comparisons one-way ANOVAs followed by the Bonferroni correction were performed. All statistics were analyzed with the program SigmaStat (Jandel Scientific).

Results

A total of 2055 eligible cases were identified (Table 1). Of these, 113 men were on metformin and had a median potential follow-up of 95.7 months. 544 men had BF and 463 deaths were recorded, of which 153 were from PC. Approximately 30% of patients had had short-term ADT (given as neoadjuvant concurrently with RT), and 25% had had long-term ADT (neoadjuvant and adjuvant). There were similar characteristics of T-stage, PC risk and ADT usage in those who were on metformin and those not on metformin. While those on metformin were of similar ages to those who were not, the overall comorbidity burden by ACE-27 score (reflected in the number of prescription medications) was higher in the metformin group (Table 1).

Metformin and outcomes

Treatment with metformin did not significantly improve time to BF, time to metastases or overall survival on a competing risk regression when adjusted for cancer risk and ACE27 (Table 2). There appeared to be a trend towards a small increase in PC-specific death in men on metformin, although the difference was not statistically significant ($p=0.08$). This finding remained unchanged on multivariate analysis (Supplementary table 1).

Further subgroup analyses of men on long-term ADT, diabetic men and by metformin dose-stratification also failed to show any significant protective effect (Supplementary tables 2 and 3). However, there was a 1.5-fold increase in the risk of PC-specific death among men treated with high-dose metformin and ADT (Table 3). A multivariate analysis adjusting for other medications confirmed this association with PC-specific deaths (Supplementary Table 4).

Metformin does not reduce HIF1 α expression or PC3 cell survival after exposure to oxidative stress.

We have previously demonstrated that HIF1 α is overexpressed in PC3 under normoxic conditions (21). Pre-treatment of PC3 cells with the mTOR inhibitor rapamycin was shown to inhibit the accumulation of HIF1 α (17). Further studies have shown that metformin inhibits mTOR via both AMP-activated protein kinase (AMPK)-dependent and -independent pathways (22). Therefore, we investigated whether metformin might reduce the expression of HIF1 α in PC3 cells via inhibition of mTOR. As shown in Fig 1A treatment of PC3 cells with increasing doses of metformin to a maximum concentration of 100 μ M had no effect on

HIF1 α . In contrast exogenous 10 μ M ferric ions (Fe²⁺) resulted in the degradation of HIF1 α to 35 \pm 7 % in PC3 cells compared to untreated control cells as demonstrated previously (14). Exposure of cells to ionizing radiation during radiotherapy generates oxidative stress via reactive oxygen species including H₂O₂. Radiotherapy and H₂O₂ have both been shown to induce cell death in PC3 cells (23), and we have previously demonstrated that overexpression of HIF1 α in PC3 under normoxic conditions contributes to resistance to oxidative stress induced by exogenous H₂O₂ (21). As seen in Fig 1B, the failure of metformin to increase the sensitivity of PC3 cells exposed to oxidative stress (50 μ M H₂O₂) is in line with the fact that metformin did not change the expression of HIF1 α . Further to test if these observations are applicable to androgen dependent prostate cancer we measured the expression of HIF1 α in LNCaP cells under normoxic conditions. The data shown in Fig 1C confirms our previous observation of a very low level of basal HIF1 α in LNCaP cells (14). Importantly metformin did not affect the expression of HIF1 α nor did it increase the sensitivity of LNCaP cells exposed to oxidative stress (25 μ M H₂O₂) as seen in Fig 1C.

Discussion

Metformin has been shown to have protective effects in PC with reduced castrate resistance and improved overall survival (6-8). A recent meta-analysis demonstrated a reduced risk of biochemical recurrence in PC (24), a finding not seen with other anti-glycaemic medications (25). Furthermore, metformin in conjunction with lifestyle changes has the added benefit of countering some of the side-effects of ADT such as reducing cardiovascular risks and metabolic syndrome (characterized by a cluster of cardiovascular risk factors) (26). However, our study failed to show an association between metformin and BF, overall survival or metastases. This is in keeping with other studies which have also failed to demonstrate any benefit from metformin in PC (10, 27-30).

A number of reasons have been suggested for these differences. One major drawback of the studies showing a clear benefit with metformin is that they have had immortal time bias, where misclassification occurred between the time of cohort entry and the exposure to metformin in a time-dependent fashion, potentially enhancing its protective effects (30). For example, Spratt et al. (2901 patients) included patients who were on metformin at the time of the diagnosis of PC or any time post radiotherapy, which included 18% of the study population who initiated metformin therapy after radiation therapy had completed (6).

Furthermore, other factors such as latency (time of exposure), reverse causality, and dose-response are also recognised biases in these studies (30). In order to overcome these biases, we examined the use of metformin only at the time of starting radiotherapy to assess the effects of metformin on treatment outcomes. However, factors such as duration of metformin use, non-compliance and undocumented variation in doses may have influenced our longer-term results.

One of the unexpected findings in our study was that treatment with higher doses of metformin was associated with a higher risk of PC-specific death in diabetic men. Although this result needs to be interpreted with caution due to the small number of prostate cancer deaths in our study, another study also reported similar findings demonstrating worse survival with metformin in diabetic men with PC (30). In that study, which corrected for the above biases associated with metformin, Bensimon et al. suggested that the poorer survival might be explained by the fact that men with clinically advanced PC are likely to be kept on oral anti-glycaemic agents such as metformin rather than converted to insulin injections as a palliative measure (30). As diabetes has been shown to increase the rate of progression of PC (27), poor glycaemic control requiring higher levels of metformin could also be an additional factor. While the small number of PC-specific mortalities (31) amongst diabetic men may explain these findings, a larger group size may be required to demonstrate statistical significance for a small effect and thus need to be interpreted with caution.

Radiotherapy and H_2O_2 have both been shown to induce apoptosis of PC3 cells through the production of reactive oxygen species (23). Metformin inhibits AMPK, thereby impacting tumour cell growth via both direct and indirect mechanisms (12). In both pathways, mammalian target of rapamycin (mTOR) plays a key role in reducing tumour cell proliferation (12). HIF1 α is a key transcription factor, regulated by mTOR, which promotes cancer growth, cell proliferation, angiogenesis, metastases and tumour cell survival (13, 14), and is also thought to increase resistance to radiotherapy (15). However, in our in vitro study, metformin failed to reduce expression of HIF1 α and correspondingly failed to further sensitise PC3 cells against cytotoxic H_2O_2 . These results are in agreement with the clinical aspect of the study, where metformin failed to show any benefit following radiotherapy. Although some previous studies have shown inhibitory effects of metformin on tumour cell proliferation or HIF1 α expression, dose response experiments indicated that the inhibitory

effect of metformin starts at 1mM and reaches a maximum at 10mM (32, 33). The small but significant adverse effect seen in patients taking high doses of metformin questions the clinical translation of these in vitro observations, although a correlation between in vitro doses of metformin with in vivo or clinical dosage has not yet been established.

In conclusion, no association was found between the use of metformin and time to metastases, time to biochemical failure or overall survival in men undergoing ADT +/- radiation therapy for PC. However, in diabetic men on high doses of metformin there may be an increased risk of PC-specific mortality. In vitro, low therapeutic concentrations of metformin had no effect on HIF1 α , and this observation could explain the conflicting evidence for metformin in men undergoing EBRT for PC. Higher, more toxic doses of metformin may be required to inhibit the mTOR-HIF1 α pathway in this patient group. Currently available data fail to provide a rationale for the use of metformin as an intervention to improve prostate cancer treatment outcomes.

Conflicts of interest

None disclosed

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Figure Legends

Figure 1: Metformin does not effect HIF1 α expression or cell survival in PC3 cells.

(A) Expression of HIF1 α protein was measured by Western blot in PC3 cells after treatment with the indicated concentration of metformin or ammonium ferric citrate (AFC) for 18 h. Protein expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Although HIF1 α protein was degraded in the presence of Fe²⁺, metformin at the indicated concentration did not reduce HIF1 α protein.

(B) PC3 cells were pre-treated with the indicated concentrations of metformin or Fe^{2+} for 4 h followed by exposure to oxidative stress ($50\mu\text{M H}_2\text{O}_2$) for 18 h as described in Materials and Methods. Cell survival was measured by MTT assay. Values are expressed as the mean \pm SEM of at least 5 independent experiments. *, $P < 0.05$ vs. untreated control; #, $P < 0.05$ vs. $50\mu\text{M H}_2\text{O}_2$.

(C) Expression of HIF1 α protein was measured by Western blot in androgen dependent cells after treatment with the indicated concentration of metformin or ammonium ferric citrate (AFC) for 18 h. Protein expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

(D) LNCaP cells were pre-treated with the indicated concentrations of metformin or Fe^{2+} for 4 h followed by exposure to oxidative stress ($25\mu\text{M H}_2\text{O}_2$) for 18 h as described in Materials and Methods. Cell survival was measured by MTT assay. Values are expressed as the mean \pm SEM of at least 5 independent experiments. *, $P < 0.05$ vs. untreated control.

Table 1: Characteristics of men using metformin vs those not on metformin.

This table summarises the characteristics of age, initial PSA (iPSA), T-stage of PC, D'Amico risk stratification of PC, duration of ADT usage, comorbidity information (Adult Comorbidity Evaluation (ACE)-27) score, type of radiation used and metformin usage. EBRT = external beam radiotherapy, HDR=high dose radiation.

Variable	Group	Metformin status		Combined N=2055
		No Metformin N=1942	Metformin N=113	
Age		70 (54 - 79)	70 (56 - 78)	70 (54 - 79)
No. Diabetic men		128	113	
iPSA		11.1 (95%CI 2.5 - 56.2)	10.7 (95%CI 0.9 - 55.2)	11.0 (95%CI 2.4 - 56.2)
Gleason Score	2-6	845 (44%)	41 (36%)	886 (43%)
	7	777 (40%)	47 (42%)	824 (40%)
	8-10	320 (16%)	25 (22%)	345 (17%)
T-Stage	1	374 (19%)	23 (20%)	397 (19%)
	2	903 (46%)	52 (46%)	955 (46%)
	3-4	665 (34%)	38 (34%)	703 (34%)
Risk	Low	233 (12%)	9 (8%)	242 (12%)
	Intermediate	779 (40%)	48 (42%)	827 (40%)
	High	930 (48%)	56 (50%)	986 (48%)
ADT	Nil	821 (43%)	58 (51%)	879 (43%)
	Short-term	600 (31%)	26 (23%)	626 (31%)
	Long-term	510 (26%)	29 (26%)	539 (26%)
Medications		2 (95%CI 0 - 7)	4 (95%CI 1 - 9)	2 (95%CI 0 - 8)
ACE-27 score	0	631 (32%)	2 (2%)	633 (31%)
	1	937 (48%)	71 (63%)	1008 (49%)
	2	317 (16%)	35 (31%)	352 (17%)
	3	57 (3%)	5 (4%)	62 (3%)
Treatment	EBRT	1536 (79%)	98 (87%)	1634 (80%)
	HDR	406 (21%)	15 (13%)	421 (20%)

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Table 2: PC outcomes for men on metformin

Treatment with metformin did not result in any apparent improvement in time to BF, time to metastases or overall survival when adjusted for cancer risk and ACE27. No significant increase in the risk of PC-specific death was observed in men on metformin.

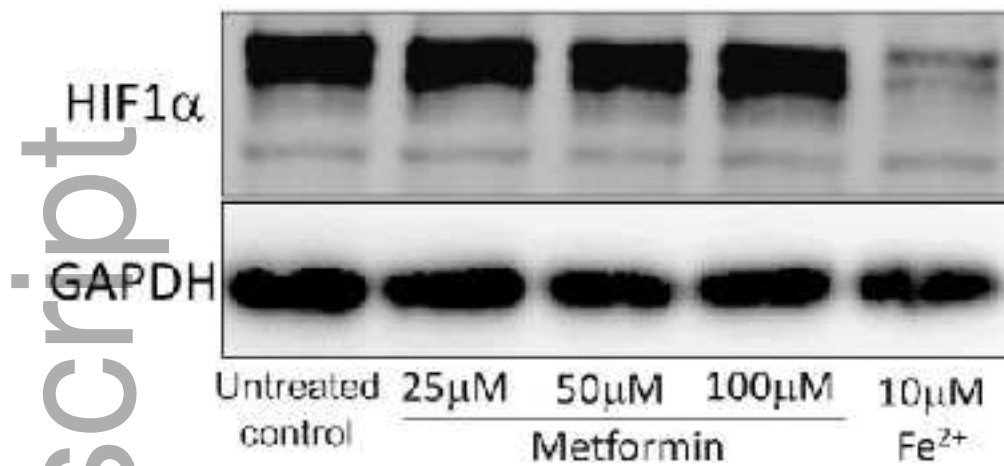
	Hazard Ratio (HR)	95% CI	P value
Biochemical failure	1.1	0.7-1.6	0.66
Metastases	1.3	0.8 – 1.9	0.26
Overall survival	1.1	0.9 – 1.4	0.32
PC-specific death	1.4	1.0 – 2.1	0.08

Table 3: PC outcomes for men on ADT with and without metformin treatment.

No beneficial effects of metformin on time to BF, time to metastases or overall survival were observed for men on ADT with metformin (n=113) or without metformin (n=997) treatment. A significant increase in the risk of PC-specific death was observed in men on metformin.

	Hazard Ratio (HR)	95% CI	P value
Biochemical failure	1.0	0.7-1.5	0.84
Metastases	1.2	1.0- 1.6	0.10
Overall survival	0.1	0.8 -1.5	0.69
PC-specific death	1.5	1.0-2.4	0.045

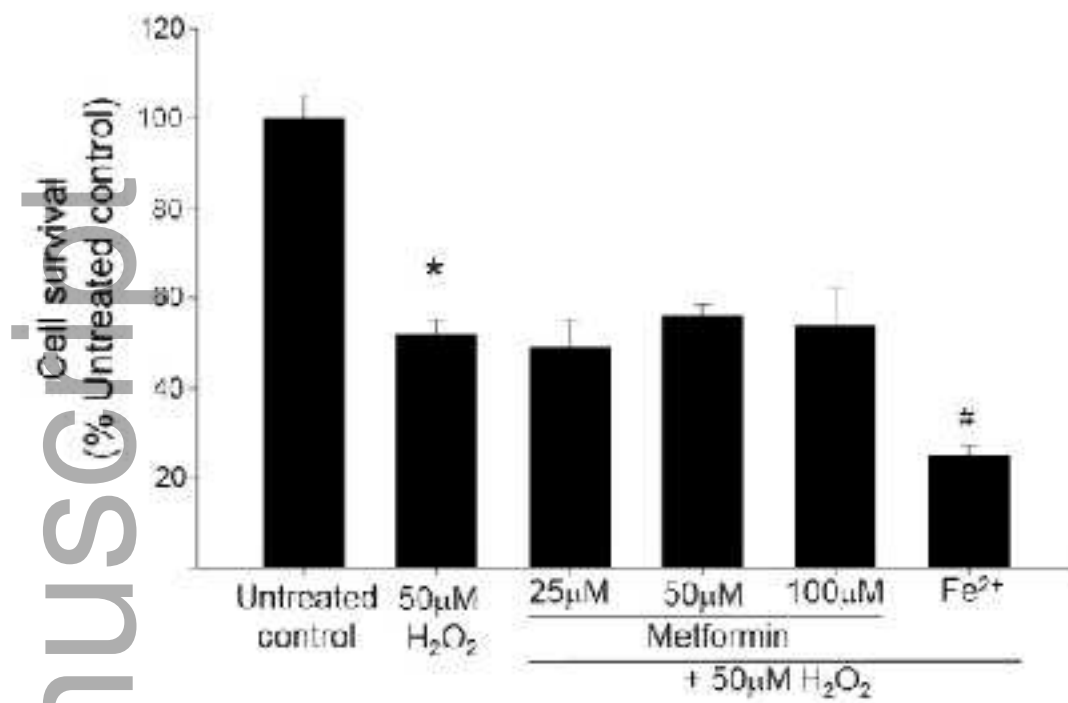
Figure 1A



bju_14709_f1a.jpg

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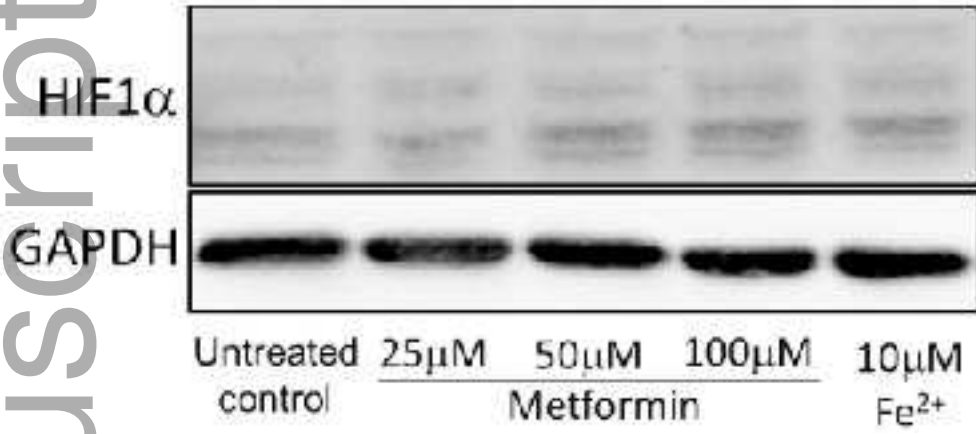
Figure 1B



bj_u_14709_f1b.jpg

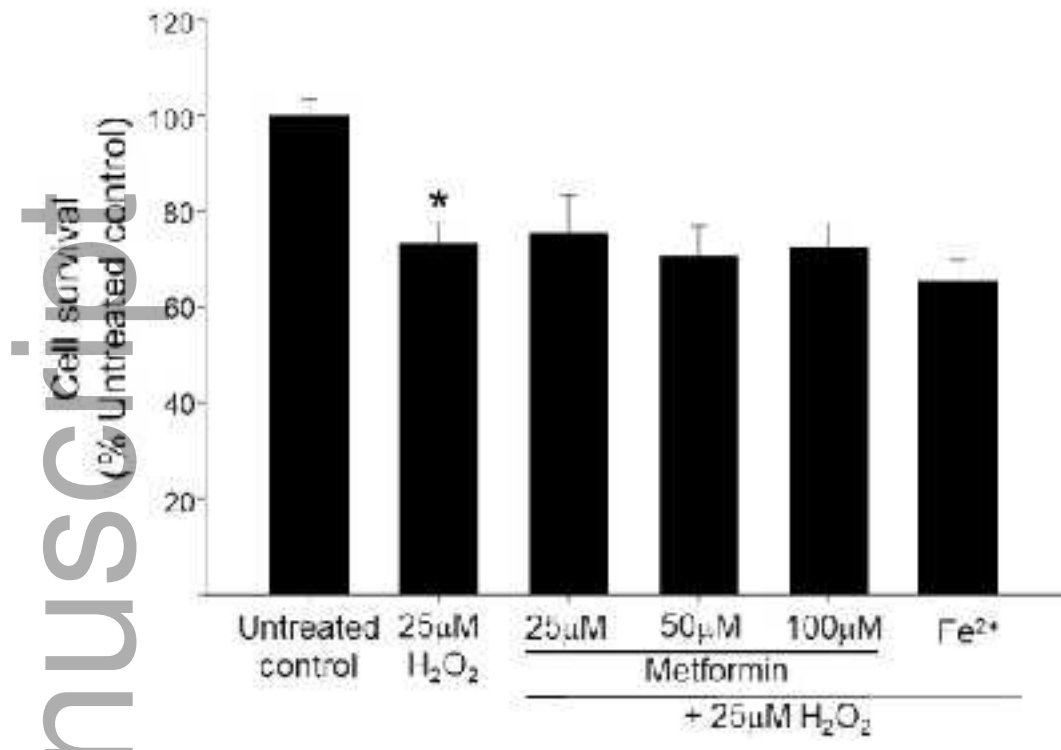
Figure 1C

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bj_u_14709_f1c.jpg

Figure 1D



bj_u_14709_f1d.jpg

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