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A Study on the Therapeutic Effect of 5-Azacytidine to Attenuate the Ramifying Repercussions of Ischemia Reperfusion Injury on Mitochondrial Molecular Machinery

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ABSTRACT

5-Azacytidine is a hypomethylating agent that has for long been used in cancer therapy due to its ability to inhibit the protein DNA methyltransferase responsible for hyper-methylating DNA strands. Recently, studies involving in vitro, ex vivo, and in vivo experiments have assessed the cardioprotective effects of 5-Azacytidine during myocardial ischemia-reperfusion injury (IRI). However, the effect of this compound in restoring the damage induced to mitochondrial molecular machinery during IRI has not yet been explored. Understanding this would help us analyze the ways through which mito-targeted therapeutics can be used. The purpose of this study is to investigate the therapeutic impact of 5-Azacytidine, as DNA methylation is a very common epigenetic modification observed during IRI. Furthermore, the protective effect of the compound in alleviating the damage induced to mitochondria during IRI can be identified, as DNA methylation can leave a direct impact on the mitochondrial genes as well. An isolated mitochondria model will be used to determine the effects of 5-Azacydine on mitochondrial molecular machinery as the capacity to generate DNA, RNA, and proteins are preserved in isolated mitochondria. In this study, we focus on the mechanisms of mitochondrial replication, and translation to understand the effect of 5-Azacytidine on the IRI affected mitochondrial system. Mitochondrial dysfunction is also another key turn of events that happens during IRI. The role of 5-Azacyidine in preserving the functionality is also being assessed in our research. The findings of these experiments would help us determine the plasticity the compound imparts on mitochondrial molecular mechanism's integrity and function post-induced IRI.

INTRODUCTION

Ischemia, which is caused by the blockage of blood vessels, is a condition that leads to infarction of tissue. The best possible treatment identified till date is reperfusion, which involves removing the obstruction to blood flow using drugs and partially or completely invasive surgery depending on the severity of the infarction. Reperfusion of tissues affected by ischemia is contraindicated in its ability to cause ischemia reperfusion injury, which is identified by certain unique pathophysiological hallmarks such as the generation of reactive oxygen species (ROS) due to the reestablishment of circulation, elevated inflammation caused by the excessive ROS, Calcium overload and mitochondrial dysfunction, which includes the abnormal opening of the MPTPs. (Frank et al., 2012; Hausenloy and Yellon, 2013; Sánchez-Hernández et al., 2020)

Researchers have been trying to understand the mechanisms and develop therapeutic measures against this condition. Recent studies implicated epigenetic modifications in regulating the expression of genes involved in pathways, contributing to IRI. A complete understanding of these epigenetic mechanisms is essential to identify an appropriate target to curb the damage caused by IRI, as a prophylactic or as a treatment option. Since they partially control gene expression patterns, these epigenetic mechanisms go beyond genetics. To better understand how ischemia reperfusion damage may be treated or prevented, the exact definition of epigenetics has been employed and controlled with the aid of inhibitors to alter gene expression patterns.

Epigenetics is the mechanism that influences heritable changes in gene expression and function without altering the genome's sequence. These epigenetic pathways are influenced by external environmental elements as well. These epigenetic mechanisms control various mediators which results in Ischemia Reperfusion Injury. Some of mediators of reperfusion injury are oxygen free radicals (ROS), endothelial dysfunction and microvascular injury, alterations in calcium level and altered myocardial metabolism. Yet these concepts, key events and the complete order of how these mechanisms can be understood is still a work in progress.

Many studies believe that targeting abnormal DNA methylation in Ischemia Reperfusion injury is a key technique for the prevention and treatment of the disease. Methylation of DNA at cytosine phosphate-guanine (CpG) dinucleotides is a typical epigenetic alteration that serves as a link between the genotype and the environment. The methyl group ($-CH_3$) is added to the 5th position of cytosine residues in Cytosine-phospho-guanine (CpG) dinucleotides during DNA methylation, resulting in chromatin condensation and gene expression changes. Enzymes known as DNA methyltransferases (DNMTs) catalyse this process, which is reversed by enzymes known as Ten-Eleven-Translocation protein

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1 (TET1), which converts 5-methyl cytosine (5mc) to 5-hydroxymethyl cytosine (5hmc). (Moore et al., 2012)
DNA methyltransferase inhibitors (DNMTi) have also been demonstrated to be useful in the treatment of ischemia-related illnesses. 5-Azacytidine, a DNMTi is a medication licensed by the US Food and Drug Administration (FDA) for the treatment of acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS), with an inhibitory impact on DNA methylation as the underlying mechanism. 5-Azacytidine has a long history of clinical use in cancer therapy. Moreover, several studies have been conducted to demonstrate the role of 5-azacytidine as a cardioprotective drug in the treatment of ischemia/reperfusion (I/R) damage. It is done in the animal model of rat. (Boovarahan & Kurian, 2021)
The discovery that 5-azacytidine was integrated into DNA and that it blocked DNA methylation when present in DNA led to its widespread use to show the relationship between the loss of methylation in particular gene areas and the activation of the related genes. (Christman, 2002) Since methylation works on DNA directly, it may work on nuclear and mitochondrial DNA. It also allows for the observation of both direct and indirect effects on epigenetic modulations on various components involved in IRI. An extensive, broad-spectrum target like methylation pattern may provide for the development of therapeutics for ischemic conditioning in the future. The main focus of this paper, will therefore be with respect to 5-Azacytidine and its therapeutic effects on ischemia reperfusion injury.

MATERIALS AND METHODS

Animals

The guidelines from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India were strictly adhered to throughout all the animal experimental procedures involved in the study. All the rights and terms has been approved by the Institutional Animal Ethical Committee (IAEC) at SASTRA Deemed to be University, Thanjavur, India for the conduct of experiments. Male Wistar Rats of weight between 250-300 grams inbred in the Central Animal Facility at SASTRA Deemed to be University, Thanjavur, India was used in the study. The rats were all housed in a well-ventilated polycarbonate cage in a temperature-controlled room at (22 ± 2°C) with a relative humidity of (60 ± 5%). The animals were exposed to 12 hours of light and dark cycle with *ad libitum* supply of water and food.

Isolation of Mitochondria

The organelle mitochondria was isolated using density gradient differential centrifugation of the organ homogenate following the guidelines from Palmer, et al. A 10% organ homogenate was prepared momentarily before the process of density gradient differential centrifugation using Isolation Buffer (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EDTA, and 0.2% BSA) with a pH of 7.4. The organ homogenate was then centrifuged at lower speed of 800g for 10 minutes at 4°C to pellet the nuclear fraction. The supernatant was then transferred into a new Eppendorf tube. The supernatant was then centrifuged at 8000g for 10 minutes at 4°C to remove any cellular debris. The supernatant was removed and the pellet was briefly resuspended in Isolation Buffer. This suspension was subjected to high centrifugation of 12,000g for 10 minutes at 4°C to pellet a pure fraction of mitochondria (Graham J. M. et al. 2002). The mitochondria was resuspended in Storage Buffer (100mM KCL, 100mM Tris-HCl, 75mM Sorbitol, 25mM Sucrose, 10mM K_2HPO_4, 5mM $MgCl_2$, 0.05mM EDTA, 0.2% BSA) at a pH of 7.4 and their protein concentration was determined with the use of Bradfords reagents (Bio-Rad). A Bovine Serum Albumin standard was used to determine the concentration. After the process of protein estimation, the isolated mitochondria were randomly divided in to groups and subjected to Normoxia and Hypoxia Reperfusion using Respiratory Buffer (300mM Mannitol, 100mM KCl, 20mM HEPES, 10mM KH_2PO_4, 5mM $MgCl_2$, 1mM EGTA, 0.2% BSA) at pH 7.1 and Hypoxia Buffer purged with N_2 (75mM NaCl, 25mM HEPES, 20mM Lactate, 16mM KCl, 10mM $NaHCO_3$, 5mM Deoxy-d-glucose, 1.2mM $MgCl_2$, 1.2mM $CaCl_2$, 1mM KH_2PO_4) at pH6.8.
Experimental Groups
Isolated mitochondria from rat hearts after normalisation were randomly divided into six groups and details of each experimental groups are as follows:
1. Normal - After the process of mitochondria isolation, the organelle was subjected to equilibration using Respiratory Buffer for 1 hour 15 minutes to maintain normal respiration.
2. Normal + 5-Azacytidine - After the process of mitochondria isolation, the organelle was subjected to equilibration using Respiratory Buffer for 15 minutes followed by 0.5 µM 5-Azacytidine pre-treatment for another 15 minutes. At the end of drug pre-treatment, the mitochondria were centrifuged at high speed of 12,000g for 10 minutes at 4°C to pellet a pure fraction of mitochondria. Then, the mitochondrial pellet was resuspended in Respiration Buffer for 45 minutes to maintain normal respiration.
3. Normal + DMSO - After the process of mitochondria isolation, the organelle was subjected to equilibration using Respiratory Buffer for 15 minutes followed by 0.5 µM DMSO pre-treatment for another 15 minutes. At the end of drug pre-treatment, the mitochondria were centrifuged at high speed of 12,000g for 10 minutes at 4°C to pellet a pure fraction of mitochondria. Then, the mitochondrial pellet was resuspended in Respiration Buffer for 45 minutes to maintain normal respiration.
4. IR - After the process of mitochondria isolation, the organelle was subjected to equilibration using Respiratory Buffer for 30 minutes followed by highspeed centrifugation at 12,000g for 10 minutes at 4°C to pellet a pure fraction of mitochondria. Then, the mitochondrial

pellet was resuspended in Hypoxia Buffer for 15 minutes to hypoxia followed by a 30 minutes incubation in Respiration Buffer to facilitate reperfusion.

5. IR + 5-Azacytidine - After the process of mitochondria isolation, the organelle was subjected to equilibration using Respiratory Buffer for 15 minutes followed by 0.5 µM 5-Azacytidine pre-treatment for another 15 minutes. At the end of drug pre-treatment, the mitochondria were centrifuged at high speed of 12,000g for 10 minutes at 4°C to pellet a pure fraction of mitochondria. Then, the mitochondrial pellet was resuspended in Hypoxia Buffer for 15 minutes to hypoxia followed by a 30 minutes incubation in Respiration Buffer to facilitate reperfusion.

6. IR + DMSO - After the process of mitochondria isolation, the organelle was subjected to equilibration using Respiratory Buffer for 15 minutes followed by 0.5 µM DMSO pre-treatment for another 15 minutes. At the end of drug pre-treatment, the mitochondria were centrifuged at high speed of 12,000g for 10 minutes at 4°C to pellet a pure fraction of mitochondria. Then, the mitochondrial pellet was resuspended in Hypoxia Buffer for 15 minutes to hypoxia followed by a 30 minutes incubation in Respiration Buffer to facilitate reperfusion.

Evaluation of Mitochondrial Function using Mitochondrial Electron Transport Chain Complex Activity

After the estimation of protein and normalisation of mitochondria, the mitochondrial electron transport chain activity was measured spectrophotometrically by employing a specific donor-acceptor oxidoreductase in a 0.1M phosphate buffer (Frazier et al. 2012). For determination of mitochondrial Complex1 activity a Rotenone sensitive NADH oxidoreductase was used. A Succinate decyl ubiquinone 2,6-dichlorophenolindophenol (DCPIP) reductase was used determine the mitochondrial Complex II activity. To determine the mitochondrial Complex III and IV activity cytochrome C reductase and cytochrome c oxidase was used as previously described by Ansari et al.

Oxidative Stress Assessment

The antioxidant profile of the isolated mitochondria homogenate was evaluated using Catalase activity, SOD activity and GSH:GSSG ratio. All the experiments used a multimode spectrophotometric plate reader to measure kinetic absorbance as well as endpoint absorbance.

A reaction buffer containing 0.1 M sodium phosphate buffer, pH 7.2, 4 mM H_2O_2, and 5 N H_2SO_4 was added to the isolated mitochondrial samples. The reaction was initiated by the addition of 0.005M $KMnO_4$ to the reaction buffer containing samples. The change in optical density was measured kinetically measured at 515 nm to assess the catalase activity (Goldblith et al. 1950).

A reaction buffer containing 45mM Tris, 1mM EDTA was added to the isolated mitochondria samples. The reaction was initiated by the addition of 2.5mM Pyrogallol to the reaction buffer containing samples. The change in optical density was measured kinetically at 420 nm to assess the superoxide dismutase activity (Nandi et al. 1988).

A reaction buffer containing 0.25m sodium phosphate and 5% Trichloroacetic acid was added to the isolated mitochondria samples. The reaction was initiated by the addition of Ellman's reagent (5,5'-dithiobis-2-nitro-benzoic acid) to the reaction buffer containing samples. The change in colour due to formation of thionitrobenzoate was measured at 412 nm to assess the GSH activity (Sedlak et al. 1968).

A reaction buffer containing 0.25m sodium phosphate buffer, 0.5mM EDTA, 4mM Oxidised glutathione and 0.2mM NADPH was added to the isolated mitochondria samples. The reaction was initiated by the addition of Ellman's reagent (5,5'-dithiobis-2-nitro-benzoic acid) to the reaction buffer containing samples. The change in optical density due to oxidation of NADPH was measured at 340 nm to assess the GSSG activity (Sedlak et al. 1968).

In vitro Mitochondrial Protein Synthesis

The experiment in vitro mitochondrial protein synthesis was carried out according to the procedures earlier by Fernandez-Silva et al. The isolated mitochondria were briefly suspended in MAITE Buffer (75mM Sorbitol, 25mM Sucrose, 10mM KCl, 10mM K_2HPO_4, 0.05mM Tris-HCl) at pH 7.4 containing 10mM Glutamate, 10mM Succinate, 2.5mM Malate, 1mM ADP and 1mg/ml of BSA. The process of translation was initiated by adding 100 µg/ml emetine, 100 µg/ml cycloheximide, and 10 µM of the 20 L-amino acids to the medium followed by an incubation for 25 minutes in gentle shaker. At the end of incubation process, mitochondria were pelleted by a high-speed centrifugation at 12000g for 10mins at 4°C. The pelleted mitochondria were then suspended in Lysis buffer (137mM NaCl, 20mM Tris-HCl, 50mM EDTA, 1% NP40) with protease inhibitors (2mM Na_2VO_4, 2mM NaF, 0.1 PMSF[Phenylmethylsulfonylfluoride]) followed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (Garrido et al., 2008).

A 12% Resolving gel was used for SDS PAGE. After the process of electrophoresis, the gels were stained using Coomassie Brilliant Blue Stain for a period of 4 hours. At the end of 4 hours, a de-staining solution was used to remove the excess stain for a period of 2 hours. The gels were then visualised using Quantity One Software (Bio-Rad, California, USA). The band intensity was quantified using ImageJ software.

In vitro Mitochondrial DNA Synthesis

The experiment in vitro mitochondrial DNA synthesis was carried out according to the procedures earlier by Fernandez-Silva et al. The isolated mitochondria were briefly suspended in MAITE Buffer (75mM Sorbitol, 25mM Sucrose, 10mM KCl, 10mM K2HPO4, 0.05mM Tris-HCl) at pH 7.4 containing 10mM Glutamate, 10mM Succinate, 2.5mM Malate, 1mM ADP and 1mg/ml of BSA. The process of translation was initiated by adding 50 µM of each dNTP to the medium followed by an incubation for 5 hours in gentle shaker. At the

end of incubation process, mitochondria were pelleted by a high-speed centrifugation at 12000g for 10mins at 4°C. The pelleted mitochondria were then suspended in Lysis buffer (150mM NaCl, 20mM Tris-HCl, 20mM EDTA, 1%SDS) at pH-8.75 in the presence of 10 µM Proteinase K and 10 µM RNase A followed by Agarose gel electrophoresis (Garrido et al., 2008).

Mitochondrial DNA Isolation and DNA Quantification

Intact mitochondrial DNA was isolated by following the exact experimentation procedure mentioned by Martia, et al. Phenol, Cholorform and Isoamyl alcohol was used for biphase separation followed by precipitation of DNA by 100% ethanol. The precipitated was washed thrice using 75% ethanol and the dried DNA pellet was dissolved in elution buffer AE (5mM Tris-Hcl) at pH 8.5. The DNA sample were then quantified using a nanodrop spectrophotometer by Thermo Fisher Scientific (NanoDrop 2000 Spectrophotometer) (Enriquez et al., 1996).

Statistical Analysis

All the statistical analysis involved through the study was carried out using Prism version 8 (Graph Pad Software Inc., San Diego, CA, USA). The data analysis was carried out using Two-way analysis of variance (ANOVA), followed by Dunnet's post-test. The experimental results were expressed as mean ± SD, and a $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

5-Azacytidine preserves the mitochondrial function during IR in an isolated mitochondrial system

Mitochondrial dysfunction is one of the key events to occur during ischemia reperfusion injury. This has an irreversible impact over the mitochondria even after various therapeutic intervention. So, an ideal therapeutic should overcome permanent mitochondrial dysfunction. Mitochondrial integrity with 5-Azacytidine pre-treatment was evaluated. From the evaluation we were able to identify that 0.5 µM 5-Azacytidine was protective against IR. So, an optimal dose 0.5 µM was fixed as standard throughout all the experimentation procedures involving isolated mitochondria. We further evaluated the electron transport chain enzyme activity to asses the mitochondrial function. The IR groups pre-treated with 5-Azacytidine has improved levels of electron transport chain activity. 5-Azacytidine pre-treatment in IR preserved mitochondrial electron transport chain complex activity of complex I, II, III and IV by %, %, % & % in the mitochondria isolated from heart, when compared to mitochondrial groups subjected to IR. 5-Azacytidine pre-treatment in IR preserved mitochondrial electron transport chain complex activity of complex I, II, III and IV by %, %, % & % in the mitochondria isolated from kidney, when compared to mitochondrial groups subjected to IR.

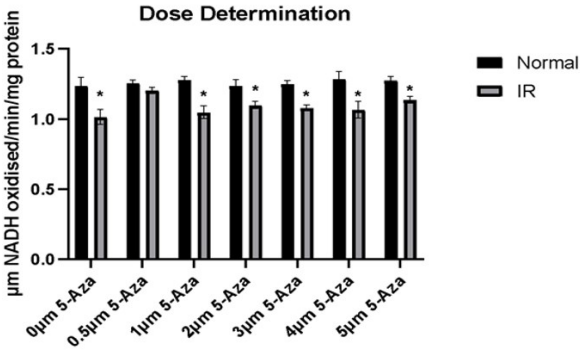


Figure 1: Dosage Determination: Optimal concentration of 5-Azacytidine drug was estimated to be 0.5 µm using NQR assay for an isolated mitochondrial system subjected to IR

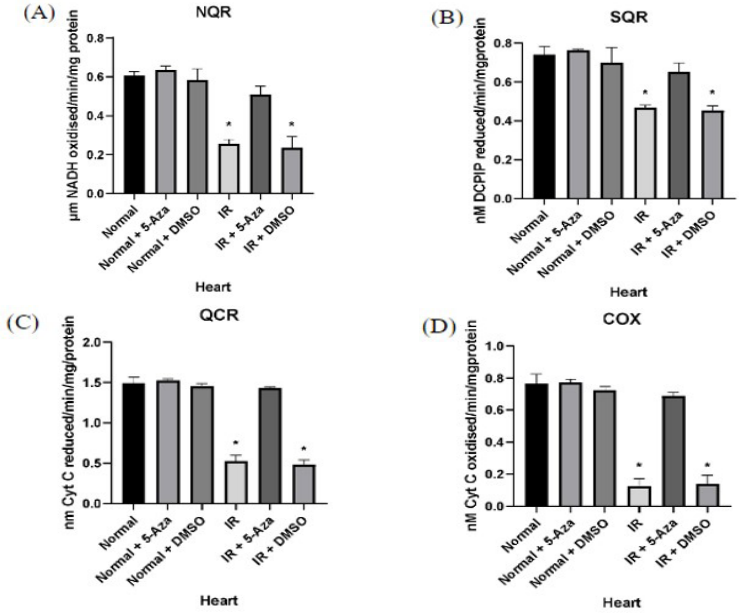


Figure 2: Effect of 5-Azacytidine on mitochondrial electron transport chain complex activity in isolated rat heart mitochondria

Effect of 5-Azacytidine on mitochondrial electron transport chain complex activity in isolated rat heart mitochondria.

(A) Complex I activity (NQR- NADH dehydrogenase) was measured as µmol NADH oxidized/min/mg protein, (B) Complex II activity (SQR- Succinate dehydrogenase) was measured in nmol DCPIP reduced/min/mg protein, (C) Complex III activity (QCR- Cytochrome bc1) was measured in nmol Cytochrome C reduced/min/mg protein, and (D) Complex IV activity (COX- Cytochrome c oxidase) was measured in nmol Cytochrome C oxidized/min/mg protein.(5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion.) Data were represented as mean ± SD. (n=6 per group). *p < 0.05 versus IR.

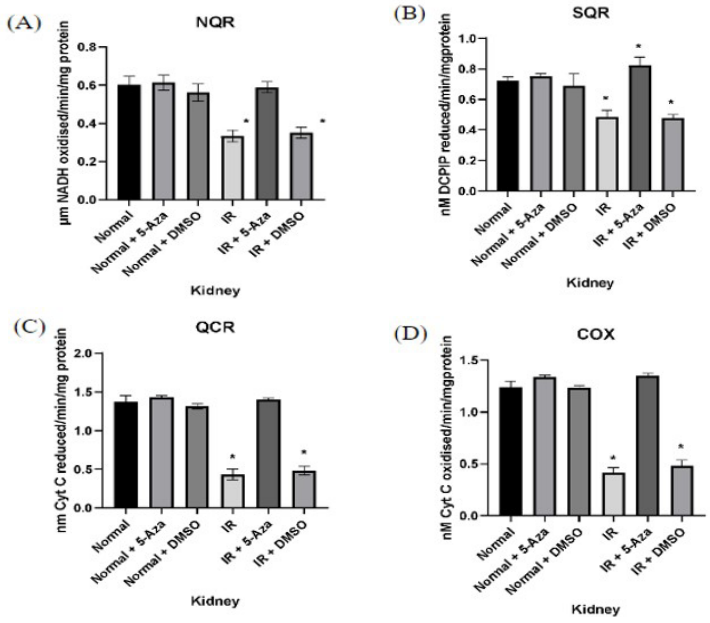


Figure 3: Effect of 5-Azacytidine on mitochondrial electron transport chain complex activity in isolated rat kidney mitochondria

Effect of 5-Azacytidine on mitochondrial electron transport chain complex activity in isolated rat kidney mitochondria.

(A) Complex I activity (NQR- NADH dehydrogenase) was measured as µmol NADH oxidized/min/mg protein, (B) Complex II activity (SQR- Succinate dehydrogenase) was measured in nmol DCPIP reduced/min/mg protein, (C) Complex III activity (QCR- Cytochrome bc1) was measured in nmol Cytochrome C reduced/min/mg protein, and (D) Complex IV activity (COX- Cytochrome c oxidase) was measured in nmol Cytochrome C oxidized/min/mg protein.(5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion.) Data were represented as mean ± SD. (n=6 per group). *p < 0.05 versus IR.

Effect of 5-Azacytidine in alleviating damage induced to mitochondria due to oxidative stress during IRI

A robust release of free radicals is one of the most critical events involved in IR injury pathology. This is also one of the key contributors to mitochondrial dysfunction. The therapeutic effect of 5-Azcytidine in overcoming and altering the damages due oxidative stress induced by IR is vital.

Mitochondrial oxidative stress parameters were assessed with 5-Azacytidine pre-treatment. From the evaluation, the IR groups pre-treated with 5-Azacytidine has improved levels of catalase and superoxide dismutase activity by % and % in the mitochondria when compared to mitochondrial groups subjected to IR, isolated from heart. 5-Azacytidine showed improved levels of catalase and superoxide dismutase activity by % and % in the mitochondria when compared to mitochondrial groups subjected to IR, isolated from kidney.

Further experimental evaluation revealed that the IR groups pre-treated with 5-Azacytidine has improved levels of GSH:GSSG ratio by % in the mitochondria when compared to mitochondrial groups subjected to IR, isolated from heart. 5-Azacytidine showed improved levels of GSH:GSSG ratio by % in the mitochondria when compared to mitochondrial groups subjected to IR, isolated from kidney.

Assessment of oxidative stress damage in cardiac mitochondria subjected to 5-Azacytidine pre-treatment.

(A) Catalase enzyme activity, (B) SuperOxide Dismutase (SOD) enzyme activity, and (C) GSH:GSSG activity; all measured in isolated mitochondrial lysates of rat kidneys. Values are represented as mean ± SD of six individual animals per group. *p < 0.05 versus IR. Abbreviation:5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion.

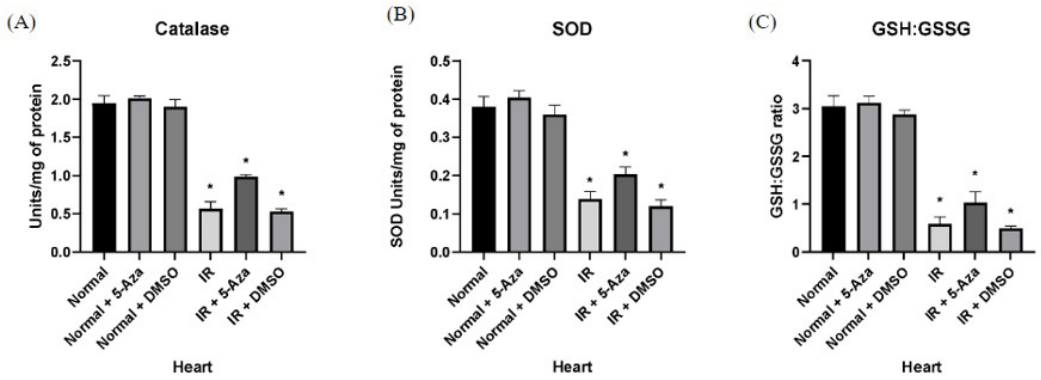


Figure 4: Assessment of oxidative stress damage in cardiac mitochondria subjected to 5-Azacytidine pre-treatmen

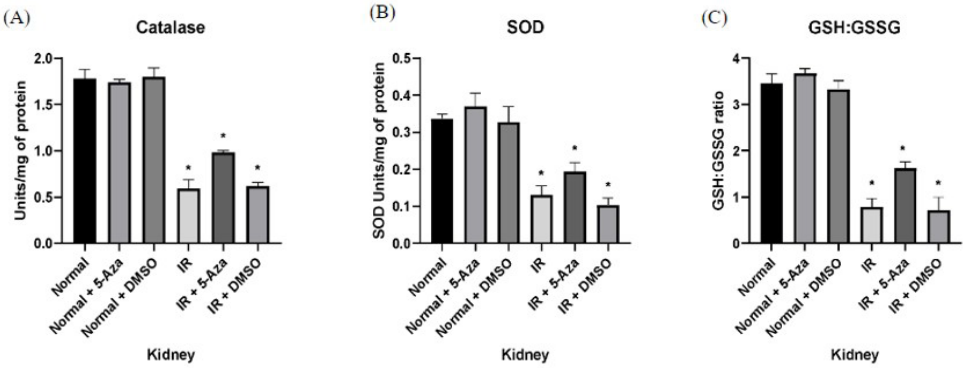


Figure 5: Assessment of oxidative stress damage in renal mitochondria subjected to 5-Azacytidine pre-treatment

Assessment of oxidative stress damage in renal mitochondria subjected to 5-Azacytidine pre-treatment. (A) Catalase enzyme activity, (B) SuperOxide Dismutase (SOD) enzyme activity, and (C) GSH:GSSG activity; all measured in isolated mitochondrial lysates of rat kidneys.. Values are represented as mean ± SD of six individual animals per group. *p < 0.05 versus IR. Abbreviation:5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion

5-Azacytidine pre-treatment overcomes impaired protein translational ability in an isolated mitochondrial system

In vitro protein translation ability of mitochondria in an isolated mitochondria system is assessed and visualised in the experiment. Epigenetic modification during IRI is one of the most reasons for translational inaccuracy. Hampered protein synthesis is one of the main reasons behind irreversible recovery from IRI. The ameliorative effect of 5-Azacytidine in overcoming translational inaccuracy when subjected is explored in our study. The significant improvement in IR groups treated with 5-Azacytidine compared to IR groups can be clearly visualised through the electrograms of the SDS PAGE gels.

Mitochondrial translational ability was assessed after 5-Azacytidine pre-treatment. Before subjecting the mitochondria from heart to in vitro protein translation, the mitochondrial protein was normalised to mg/ml across all groups. After the process of in vitro translation, the IR groups pre-treated with 5-Azacytidine showed an increase in mitochondrial protein by % when compared to mitochondrial groups subjected to IR in heart.

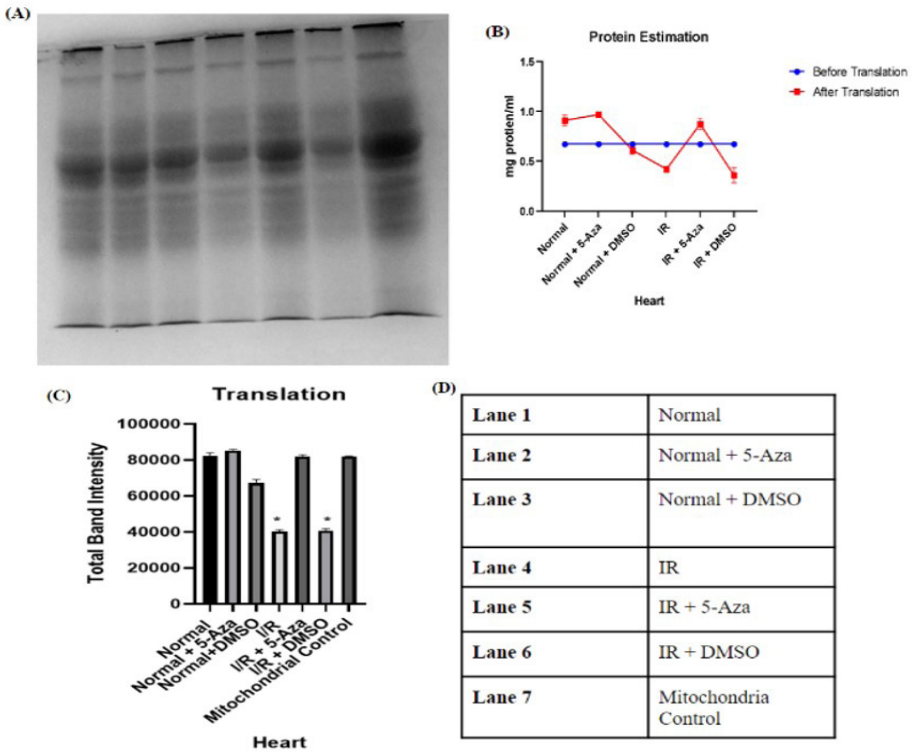
Before subjecting the mitochondria from kidney to in vitro protein translation, the mitochondrial protein was normalised to mg/ml across all groups. After the process of in vitro translation, the IR groups pre-treated with 5-Azacytidine showed an increase in mitochondrial protein by % when compared to mitochondrial groups subjected to IR in kidney.

Table 1: Quantitative Mitochondrial Protein Estimation by Bradfords Method in Heart Tissue Before In Vitro Protein Translation

S.No	Group Name	Protein	Unit
1.	Normal	0.67333333	mg/ml
2.	Normal + 5-Aza	0.67333333	mg/ml
3.	Normal + DMSO	0.67333333	mg/ml
4.	IR	0.67333333	mg/ml
5.	IR + 5–Aza	0.67333333	mg/ml
6.	IR+DMSO	0.67333333	mg/ml

Table 2: Quantitative Mitochondrial Protein Estimation by Bradfords Method in Heart Tissue after in Vitro Protein Translation

S.No	Group Name	Protein	Unit
1.	Normal	0.909	mg/ml
2.	Normal + 5-Aza	0.97	mg/ml
3.	Normal + DMSO	0.612	mg/ml
4.	IR	0.42	mg/ml
5.	IR + 5–Aza	0.873	mg/ml
6.	IR+DMSO	0.359	mg/ml



Lane 1	Normal
Lane 2	Normal + 5-Aza
Lane 3	Normal + DMSO
Lane 4	IR
Lane 5	IR + 5-Aza
Lane 6	IR + DMSO
Lane 7	Mitochondria Control

Figure 6: In-vitro cardiac mitochondrial translated proteins analysis using SDS-PAGE gel electrophoresis

In-vitro cardiac mitochondrial translated proteins analysis using SDS-PAGE gel electrophoresis.
(A) SDS PAGE gel electrogram, (B) Protein estimation, (C) Total Band Intensity, and (D) Representations for gel electrogram lanes. Values are represented as mean ± SD of six individual animals per group. *p < 0.05 versus IR. (5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion.)

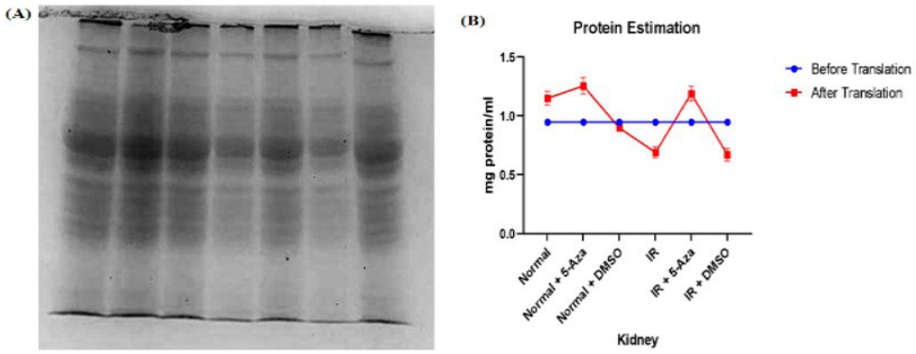
Table 3: Quantitative mitochondrial protein estimation by Bradfords method in kidney tissue before in vitro protein translation

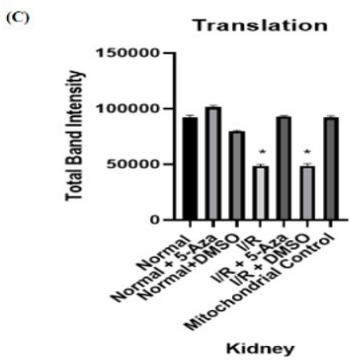
S.No	Group Name	Protein	Unit
1.	Normal	0.94833333	mg/ml
2.	Normal + 5-Aza	0.94833333	mg/ml
3.	Normal + DMSO	0.94833333	mg/ml
4.	IR	0.94833333	mg/ml
5.	IR + 5–Aza	0.94833333	mg/ml
6.	IR+DMSO	0.94833333	mg/ml

Table 4: Quantitative mitochondrial protein estimation by Bradfords method in kidney tissue after in vitro protein translation

S.No	Group Name	Protein	Unit
1.	Normal	1.1505	mg/ml
2.	Normal + 5-Aza	1.2545	mg/ml
3.	Normal + DMSO	0.9008	mg/ml
4.	IR	0.69	mg/ml
5.	IR + 5–Aza	1.1896	mg/ml
6.	IR+DMSO	0.67087	mg/ml

In-vitro renal mitochondrial translated proteins analysis using SDS-PAGE gel electrophoresis.
(A) SDS PAGE gel electrogram, (B) Protein estimation, (C) Total Band Intensity, and (D) Representations for gel electrogram lanes. Values are represented as mean ± SD of six individual animals per group. *p < 0.05 versus IR. (5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion.).





(D)

Lane 1	Normal
Lane 2	Normal + 5-Aza
Lane 3	Normal + DMSO
Lane 4	IR
Lane 5	IR + 5-Aza
Lane 6	IR + DMSO
Lane 7	Mitochondria Control

Figure 7: In-vitro renal mitochondrial translated proteins analysis using SDS-PAGE gel electrophoresis

5-Azacytidine enhances DNA replication machinery in an isolated mitochondrial system

In vitro DNA replication ability of mitochondria in an isolated mitochondria system is assessed and visualised in the experiment. DNA hypermethylation during IRI is one of the most reasons for transcriptional impairment and translational inaccuracy. Upregulation of DNMT1 during IR is one of the major reasons behind extensive and far-fetched damage. The therapeutic effect of 5-Azacytidine in overcoming impaired DNA replication machinery when subjected is explored in our study. The significant enhancement in mitochondrial DNA copy number in IR groups treated with 5-Azacytidine compared to IR groups can be clearly visualised through the electrograms of the Agarose gels.

Mitochondrial DNA replication ability was assessed after 5-Azacytidine pre-treatment. Before subjecting the mitochondria from heart to in vitro DNA replication, the mitochondrial protein was normalised to mg/ml across all groups. After the process of in vitro DNA replication, the IR groups pre-treated with 5-Azacytidine showed an increase in mitochondrial DNA by % when compared to mitochondrial groups subjected to IR in heart.

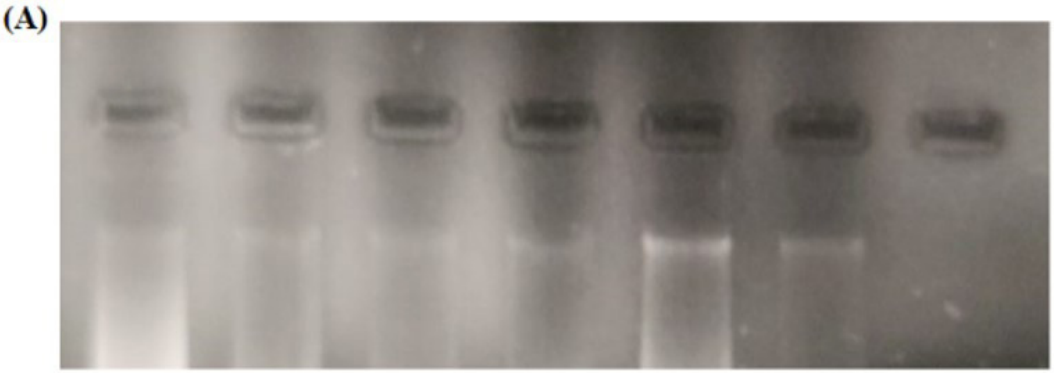
Before subjecting the mitochondria from kidney to in vitro DNA replication, the mitochondrial protein was normalised to mg/ml across all groups. After the process of in vitro DNA replication, the IR groups pre-treated with 5-Azacytidine showed an increase in mitochondrial DNA by % when compared to mitochondrial groups subjected to IR in kidney.

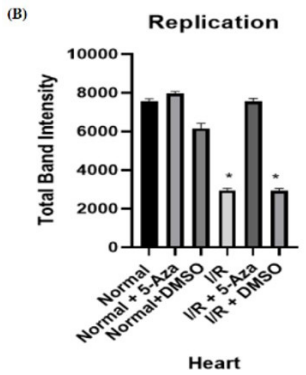
Table 5: Quantitative mitochondrial protein estimation by Bradfords method in heart tissue before in vitro DNA replication

S.No	Group Name	Protein	Unit
1.	Normal	0.763	mg/ml
2.	Normal + 5-Aza	0.763	mg/ml
3.	Normal + DMSO	0.763	mg/ml
4.	IR	0.763	mg/ml
5.	IR + 5–Aza	0.763	mg/ml
6.	IR+DMSO	0.763	mg/ml

Table 6: Mitochondrial DNA quantification in Heart using NanoDrop Spectrophotometer after in vitro DNA replication

S.No	Group Name	Nucleic acid	Unit	260/280	260/230	Sample Type
1.	Normal	578.25	ng/µl	1.81	2.12	DNA
2.	Normal + 5-Aza	583.14	ng/µl	1.82	2.14	DNA
3.	Normal + DMSO	462.69	ng/µl	1.9	2.11	DNA
4.	IR	258.18	ng/µl	1.9	2.12	DNA
5.	IR + 5–Aza	565.82	ng/µl	1.89	2.17	DNA
6.	IR+DMSO	264.28	ng/µl	1.83	2.0	DNA





(C)

Lane 1	Normal
Lane 2	Normal + 5-Aza
Lane 3	Normal + DMSO
Lane 4	IR
Lane 5	IR + 5-Aza
Lane 6	IR + DMSO

Figure 8: Agarose gel electrophoresis analysis of In-vitro cardiac mitochondrial DNA replication

Agarose gel electrophoresis analysis of In-vitro cardiac mitochondrial DNA replication.
(A) Agarose gel electrogram, (B) Total Band Intensity, and (C) Representations for gel electrogram lanes. Data were represented as mean ± SD. (n=6 per group). *p < 0.05 versus IR. Abbreviation:5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion.

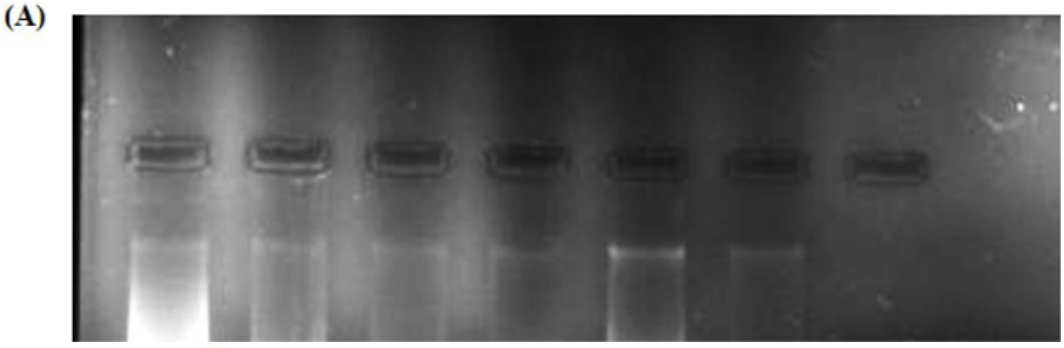
Agarose gel electrophoresis analysis of In-vitro renal mitochondrial DNA replication.
(A) Agarose gel electrogram, (B) Total Band Intensity,

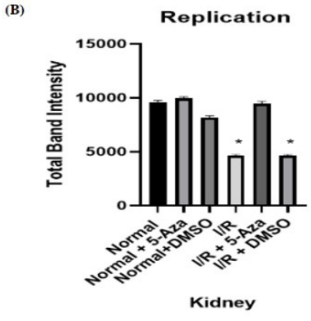
Table 7: Quantitative mitochondrial protein estimation by Bradfords method in kidney tissue before in vitro DNA replication

S.No	Group Name	Protein	Unit
1.	Normal	0.984	mg/ml
2.	Normal + 5-Aza	0.984	mg/ml
3.	Normal + DMSO	0.984	mg/ml
4.	IR	0.984	mg/ml
5.	IR + 5–Aza	0.984	mg/ml
6.	IR+DMSO	0.984	mg/ml

Table 8: Mitochondrial DNA quantification in Kidney using NanoDrop Spectrophotometer after in vitro DNA replication

S.No	Group Name	Nucleic acid	Unit	260/280	260/230	Sample Type
1.	Normal	578.25	ng/µl	1.89	2.0	DNA
2.	Normal + 5-Aza	583.14	ng/µl	1.82	2.1	DNA
3.	Normal + DMSO	462.69	ng/µl	1.91	2.1	DNA
4.	IR	258.18	ng/µl	1.8	2.12	DNA
5.	IR + 5–Aza	565.82	ng/µl	1.83	2.17	DNA
6.	IR+DMSO	264.28	ng/µl	1.9	2.0	DNA





(C)

Lane 1	Normal
Lane 2	Normal + 5-Aza
Lane 3	Normal + DMSO
Lane 4	IR
Lane 5	IR + 5-Aza
Lane 6	IR + DMSO

Figure 9: Agarose gel electrophoresis analysis of In-vitro renal mitochondrial DNA replication

and (C) Representations for gel electrogram lanes. Data were represented as mean ± SD. (n=6 per group). *p < 0.05 versus IR. Abbreviation:5-Aza,5-Azacytidine; DMSO, Dimethyl sulfoxide; IR, Ischemia-reperfusion

DISCUSSION

Ischemia reperfusion injury is one of the most common and unavoidable forms of injury to occur during a revascularisation procedure or because of therapeutic interventions to an atherosclerotic plaque or an ischemic stroke (Piper et al., 1998). The injury might seem inevitable because of huge number of variable complications arising after interventional therapy. Till date, a lot of different therapeutics have been tested, repurposed and trailed for the treatment of IRI but a lot of them fails in one or many concern. In previous study by Rahavi, et al., has reported the ameliorative effect of 5-Azacytidine against IRI preliminarily due to its inherent ability to combat the DNMT1 by inhibiting this action (A.A.Mangoni et al. 2018). The role of 5-Azacytinde in combating cancer is also well explored. But, the role of 5-Azacytidine in alleviating mitochondrial damage due to IRI is not explored. As mitochondria is one of the key players in regulation and resuscitating cell survival during IRI, it is of immense importance to have a deep understanding of the mechanism through which 5-Azacytidine helps alleviating IRI. The mechanistic action on drug on mitochondria is not well documented and is unexplored. So, having a better understanding of the way through which mitochondria mediates IR protection would help enable a complete overlay (D.Jain et al. 2017). Even though there are multiple drugs to treat IRI, such multifaceted view on the action of drug is absent thereby most drugs fail to transition even into clinical trials. Therefore, phrenological manipulative ability without enough substantial evidence is not fruitful yield (I.Andreadou et al. 2020).

Many drugs like methotrexate, hydroxychloroquine which are used for the treatment of various diseases like cancer, malaria have been repurposed to treat cardiovascular diseases as well (A.Daiver et al. 2021). 5-Azacytidine mediated epigenetic reprogramming is used extensively in cancer research. The mechanism of action and epigenetic modulation mediated by 5-Azacytidine is clearly established and well documented (E.Hervout et al. 2013). Also, many studies have reported the cardioprotective, nephroprotective and vasculo-protective nature of the compound. But the effect of this compound on cardiac IRI and renal IRI are not well established (S.Sou et al, 2016). Therefore, a proper evidence-based study would help us repurpose the drug effectively to manage many cardiovascular and renal complications in a clinical scenario as a potent pharmacological agent (L.Badimon et al. 2019). From our study, 5-Azacytidne has been to shown to provide an ameliorative effect against IRI in an Isolated Mitochondrial system thereby, providing a promising role as a pharmacological intervention.

The organs heart and kidney have been shown to exhibit an elevated levels of mitochondrial dysfunction because of the free radical stack and extensive calcium overload (H.Ma et al. 2011). This immediately results in the change in functionality of the mitochondria leading to loss of mitochondrial bioenergetics (K.Yang et al. 2017). Based on these evidences, we identified a significantly decreased mitochondrial electron transport chain activity due to deterioration in function of mitochondrial complexes I, II, III, IV. There was also decreased tendency to scavenge the free radical resulting increased oxidative stress. Our studies have shown that pre-treatment with 5-Azacytidine resulted in improved mitochondrial electron transport chain activity. The results have also indicated that the scavenging effect of 5-Azacytidine but it was not that considerable when compared to its other therapeutic effect. However, the compound enhanced the survival key for mitochondria when subjected to IRI by considerably enhancing the mitochondrial functionality and improved resistance to oxidative stress induced mitochondrial damage.

Translational inaccuracy and reduced mitochondrial copy number are another key deteriorative feature of IRI. So, a pharmacological agent that has the ability to modify the epigenetics of a cellular system is vital (P.E.Nikolaou et al. 2019). Often times we fail to realise that mitochondria is a system of its own and it has a trivial role to play in cellular epigenetics as well (B.A.Hemmings et al. 2015). When subjected to IRI, the levels of mitochondrial protein translation were significantly reduced and it also resulted in decreased mitochondrial copy number. However, once the groups were pre-treated with 5-Azacytidine and when subjected to IRI hey showed improved to resistance to translational inaccuracy by enhanced protein synthesis. The epigenetic modification that 5-Azacytidine imparts on the DNA replication machinery also resulted improved mitochondrial copy number even when to IRI. Based on the results obtained from these independent experiments throughout the long run we could demonstrate the therapeutic effect of 5-Azacytidine to Attenuate the Ramifying Repercussions of Ischemia-Reperfusion Injury on Mitochondrial Molecular Machinery.

CONCLUSION

Ischemia reperfusion injury mediated mitochondrial damage is inevitable and without proper pharmacological intervention it can be irreversible. This study shows the therapeutic effect of 5-Azacytidine in attenuating IRI mediated mitochondrial dysfunction. 5-Azacytidine pre-treated groups showed improved mitochondrial translational accuracy and increased mitochondrial copy number. These evidences indicated the rooted ability of 5-Azacytidine to act on mitochondria to ameliorate IRI induced mitochondrial damage and thereby contributing towards cellular homeostasis.

FUTURE PROSPECTS

From our findings pre-treatment with 5- Azacytidine has enhanced the ability of protein translation, increased

mitochondrial copy number and have restored the damage caused to mitochondrial function when subjected to ischemia reperfusion injury in an isolated mitochondrial system. An isolated mitochondrial system acts as great tool in understanding the specificity, targeted action and therapeutic potential of 5-Azacytidine. However, this system doesn't exactly replicate environment and interactions of an organ system, whereas it replicates the environment of the cell to sustain a healthy mitochondria population. So, extensive animal studies consisting animals pre-treated with 5-Azacytidine are required to further evaluate the effect of 5-Azacytidine on mitochondria wherein a lot of other factors can interplay to contribute, impair or enhance the therapeutic effect. Also, the effect of 5-Azacytinde on mitochondrial gene expression associated with ischemia reperfusion and need to be studied. Furthermore, studies can also be extended to analyse the efficacy of 5-Azacytidine in attenuating any repercussions on distant organ due to ischemia reperfusion injury.

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