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Quantitative Proteomic Assessment of Key Proteins Regulated by Electrical Pulse-mediated Galloflavin Delivery in Triple-Negative Breast Cancer Cells

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Abstract: Triple-negative breast cancer (TNBC) is the most lethal subset of breast cancers, lacking targeted therapies. There is a critical need to identify alternative treatments for TNBC. Towards this, in this research, we investigated the anticancer effects of Galloflavin (GF), an LDHB inhibitor, in reducing the proliferation of MDA-MB-231, the human triple-negative breast cancer cells. To enhance the uptake of GF, we applied electrical pulses (EP) with GF and studied its protein profile characteristics and viability. We used GF at a concentration of 100µM with 800V/cm, 100µs, and eight electrical pulses to treat these cells. Label-free, high throughput, quantitative proteomics results indicated that 172 proteins were significantly downregulated, while 222 proteins were significantly upregulated. The upregulated proteins include Cytochrome C Oxidase Assembly Factor and Mitochondrial Ribosomal proteins. Key downregulated proteins include LDHB, and ENO1 in EP+GF treatments, compared to GF only, indicating the effect of EP+GF combination in reducing the proliferation of the TNBC cells. These results pave the path for additional therapy for TNBC and the various pathways the TNBC cells proceeded with Electrochemotherapy.

Keywords: proteomics; triple-negative breast cancer; Galloflavin; MDA-MB-231.

1. Introduction

Cancer is one of the world's top causes of human death[1]. Approximately 19.3 million people were diagnosed with cancer, with about 10 million deaths in 2021 worldwide [2, 3]. Out of these, breast cancer was the most common, with about 2.3 million new cases [2]. Breast cancer patients are initially treated with chemotherapy followed by radiation and surgery to eradicate the cancer cells [4]. There are cases where chemotherapy is utilized as a primary form of treatment, where cancer has already spread and cannot be surgically removed [5]. Most chemotherapeutics function as energy blockers for malignant cells in order to induce cell death [6–9]. Triple-negative breast cancer (TNBC) is aggressive in progression and they channel through alternative energy-producing pathways to sustain and develop drug resistance [10]. There is a need for alternate physical treatments, such as electroporation to enhance drug uptake and overcome drug resistance.

In a process known as electroporation, the controlled local administration of the electric field opens up porous layers in the cells, temporarily converting the membrane-permeable into

an otherwise hydrophilic membrane [11–14]. This local administration of electric pulses will increase the membrane's potential, and pore formation occurs at a high intensity of 0.5V or more [15]. This temporary permeability causes and allows the drug to reach the tumor cells better, and such therapy is termed Electrochemotherapy (ECT) [16]. ECT does not rely on the cell receptors to enhance drug delivery and influence cellular signaling [17–21]. The combined effect of LDH inhibitor and electrochemotherapy is studied in our research to provide an effective alternative to traditional cancer treatment methods and to know about the regulated proteins.

The protein profiles were studied using label-free quantitative proteomics that provides insight into the key proteins and genes, which were the combination of electrical pulses and GF vs. GF alone significantly upregulated and downregulated. Proteomics studies enabled us to screen thousands of proteins at once, providing a comprehensive understanding of the phenomenon by identifying multiple pathways used by TNBC cells. The use of proteomics to identify the various signaling effects induced by ECT with GF can be a useful strategy. The mechanistic understanding of the effects induced by ECT may aid in advancing this technique in clinical applications by eliminating off-targets. [22–24]. The viability studies conducted corroborate the proteomics study.

2. Materials and Methods

Cell culture, drug preparation, and electric pulses applied are the same as [25]. In brief, MDA-MD-231, an epithelial, human TNBC cell line, was used for the study. Cultured MDA-MB-231 cells were treated with GF only (100µM concentration) and a combination of EP+GF. 8000V/cm, 100µs, pulses were applied using BTX ECM 830 pulse generator, and the cells were incubated for 12h period and extracted for proteomics study as before [25]. Pathways and grouped proteins were analyzed using the KEGG database, with DAVID 6.8. [26]. The Gene Ontology was studied using the GeneCodis Bioinformatics tool [27]. STRING [28] was used to visualize the interaction and functional enrichment of the various proteins. Statistical analyses were also done as before.

3. Results and Discussion

Figure 1 illustrates the Venn diagram. A total of 3233 proteins were identified, of which 2466 proteins were common in both treatments; there are 397 unique proteins for GF only, while the unique proteins for EP+GF are 370. This indicates that the EP+GF treatment deleted the 397 unique proteins of GF only treatment and created 370 new unique proteins.

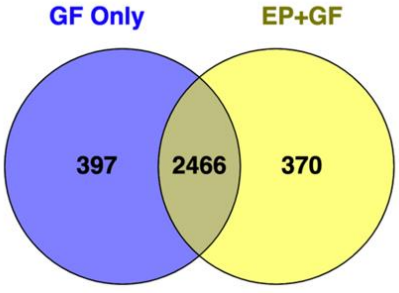

Figure 1. Venn diagram showing the distribution of proteins in different groups identified from LC-MS/MS proteomics data.

Figure 2 shows the heatmap of the protein distribution of the GF only and EP+GF samples. This heatmap was used to visualize the expression of these proteins across triplicates for GF only and EP+GF treatments. The entire linkage approach was used to group the proteins. Each treatment's triplicated proteins were grouped to exhibit consistent expression patterns. The GF and EP+GF treatment protein clusters are contrasting, indicating that the GF and EP+GF treatments generate different levels of protein expressions. It is seen that there was more downregulation seen in EP+GF treatment compared to GF only, where the red color indicates negative fold change, representing significantly downregulated proteins, and the green color indicates positive fold change, corresponding to significantly upregulated proteins. There were 172 significantly downregulated proteins and 222 significantly upregulated proteins.

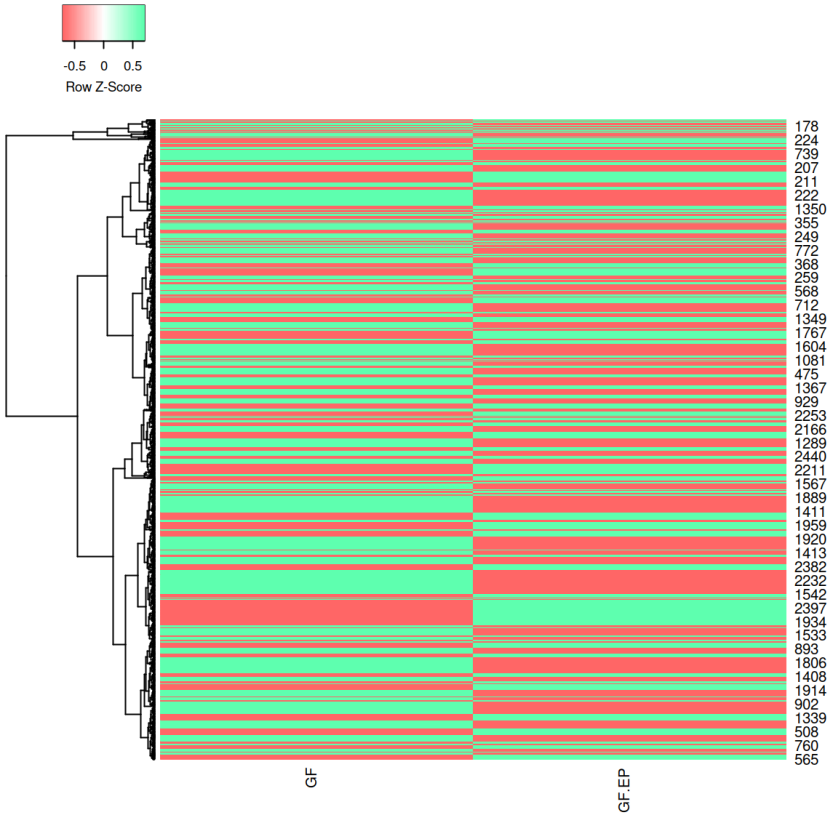

Figure 2. Heatmap of significantly upregulated and downregulated proteins.

Table 1 displays the top 15 significantly upregulated proteins and genes obtained from the proteomics study, along with their fold changes. These include various proteins, such as Cytochrome C Oxidase Assembly Factor COX2, Heat Shock Protein-HSPA14, and Ribosomal Protein-RPS28.

Table 1. Top 15 upregulated proteins and genes along with their fold change.

Protein ID	Protein Names	Gene Names	Fold Change
O15230	Laminin subunit alpha-5	LAMA5	2.13238936
A8K607	Exportin-7	XPO7	0.88262337
P33527	Multidrug resistance-associated protein 1	ABCC1	0.82424128
Q9P035	Hydroxyacyl-CoA dehydratase 3	HACD3	0.7799141
Q14XT3	Cytochrome c oxidase subunit 2	COX2	0.77427751
Q6NX51	Exocyst complex component 4	EXOC4	0.77100302
C9JZL8	Myeloid differentiation marker	MYADM	0.75425273
Q0VDF9	Heat shock kDa protein 14	HSPA14	0.74712288
B3KTQ2	Lactadherin	MFGE8	0.71623043
B2R4R9	40S ribosomal protein S28	RPS28	0.69268683

Protein ID	Protein Names	Gene Names	Fold Change
Q14789	Golgin subfamily B member 1	GOLGB1	0.66528009
A0A024R2K1	Ras-related protein Rab-5A	RAB5A	0.65661394
Q8TC07	TBC1 domain family member 15	TBC1D15	0.64874236
P09914	Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	0.62973872
Q5JVZ5	Engulfment and cell motility protein 2	ELMO2	0.62777649

Table 2 displays the top 15 significantly downregulated proteins and genes obtained from the proteomics study, along with their fold changes. The downregulated proteins include Death-inducer obliterator 1, Translation machinery-associated protein.

Table 2. Top 15 downregulated proteins and genes along with their fold change.

Protein	Protein Names	Gene Name	Fold Change
Q71DI3	Histone H3.2	HIST2H3A	-1.8139684
A0A024R306	Translation machinery-associated protein	CCDC72	-0.7811588
Q9BTC0	Death-inducer obliterator 1	DIDO1	-0.7338681
Q5RHS7	Transition metal ion binding protein	S100A2	-0.7205462
A0A024R525	Ferritin	FTH1	-0.7089763
Q6PJ77	Transcription factor BTF3	BTF3L4	-0.6907547
P63218	Guanine nucleotide-binding protein	GNG5	-0.6702852
Q8NI27	THO complex subunit 2	THOC2	-0.6428783
B8ZZQ6	Prothymosin alpha	PTMA	-0.6221542
P41208	Centrin-2	CETN2	-0.6148624
A0A024R5U3	cAMP-regulated phosphoprotein 19	ARPP-19	-0.6118163
E9PQY2	Prefoldin subunit 4	PFDN4	-0.6064312
A0A024R0Q0	Protein SMG9	FLJ12886	-0.5950033
A0A024R2K4	Leucine-rich repeat flightless-interacting protein 2	LRRFIP2	-0.5889966
A0A024R7S3	Clathrin light chain B	CLTB	-0.5591009

3.1. Upregulated pathways.

Analysis of differentially expressed proteins was established to ratify the upregulation and downregulation of pathways, which affect the cell cycle in TNBC cells. Figure 3 shows the upregulated pathways with the corresponding number and percentage of proteins upregulated in EP+GF treatment compared to GF.

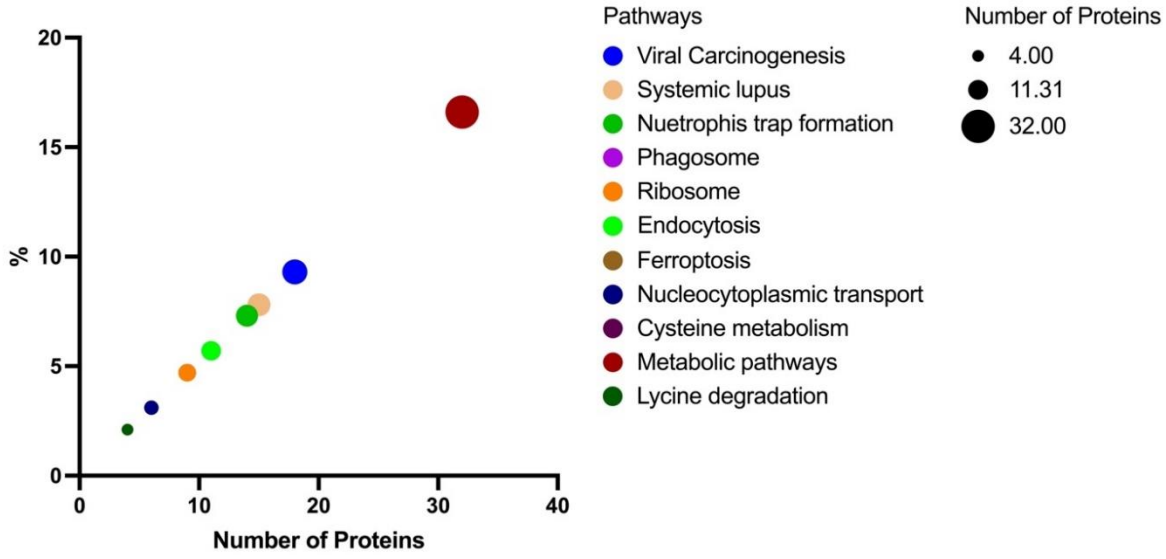

Figure 3. Upregulated pathways with the corresponding number and percentage of proteins upregulated in EP+GF treatment.

One of the significantly upregulated pathways is Ribosomal Pathways. This pathway shows the various mitochondrial ribosomal proteins (MRP) and ribosomal proteins (RP) that were significantly upregulated in EP+GF treatment compared to GF only. They include MRPL22 and MRPL3, RPL17, RPL18, RPL9, RPS24, RPS28, and RPS9. The significant upregulation of ribosomal proteins establishes the DNA damage to the TNBC cells by the treatments, and the function of mitochondrial ribosomes is to aid protein synthesis within mitochondrial, which supports producing ATP and conversion of energy [29]. Table 3 shows the list of identified proteins and genes. Figure 4 displays the String interactions of these mitochondrial ribosomal proteins along with LDHB.

Table 3. Upregulated proteins and genes in ribosomal subunits.

Protein	Gene Name	Gene ID
E7ESL0	Mitochondrial ribosomal protein L22	MRPL22
H0Y9G6	Mitochondrial ribosomal protein L3	MRPL3
A0A087WXM6	Ribosomal protein L17	RPL17
Q0QEW2	Ribosomal protein L18	RPL18
C9J4Z3	Ribosomal protein L37a	RPL37A
Q53Z07	Ribosomal protein L9	RPL9
E7ETK0	Ribosomal protein S24	RPS24
B2R4R9	Ribosomal protein S28	RPS28
A0A024R4M0	Ribosomal protein S9	RPS9

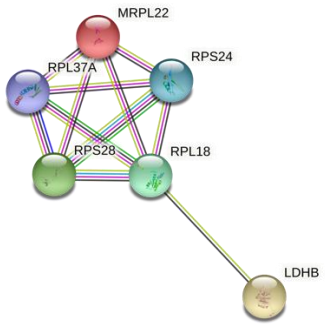

Figure 4. String interactions between upregulated mitochondrial ribosome proteins with LDHB.

In addition, endocytic pathways were also significantly upregulated. Endocytosis is one of the mechanistic pathways which are responsible for cell regulation. They involve processes like pinocytosis and phagocytosis. Here, EH domain-containing EHD4, retromer complex component (VPS35), adaptor-related protein complex 2 alpha 1 subunit (AP2A1), Golgi brefeldin A resistant guanine nucleotide exchange factor 1 (GBF1), transferrin receptor (TFRC) were upregulated by the EP+GF treatment, compared GF only. The upregulation of endocytosis pathways helps in cell cycle regulation since "endocytosis is an attenuator of signaling, and therefore a potential candidate as a tumor suppressor pathway/System" [30].

Along with the endocytic pathway, EP+GF treatments significantly increased phagosomes and phagocytosis. Immune response and the fight against malignant cells are constituted by antigen-presenting cells in the presence of Natural Killer cells. This reaction activates the adaptive immune system. Antigen-presenting cells can engulf tumor cells through phagocytosis. RAB5C, a member of the RAS oncogene family, dynein cytoplasmic 1 heavy chain 1 (DYNC1H1), integrin subunit alpha 2 (ITGA2), thrombospondin 1 (THBS1), transferrin receptor (TFRC) were some of the upregulated genes in this pathway. Upregulated proteins and genes identified in the Endocytic pathway are shown in Table 4.

Table 4. Upregulated proteins and genes identified in the endocytic pathway.

Protein ID	Gene Name	Gene ID
A8K9B9	EH domain containing 4	EHD4
A0A024R2K1	Member RAS oncogene family	RAB5A
A0A024RB09	Member RAS oncogene family	RAB5B
A0A024R1U4	Member RAS oncogene family	RAB5C
O75436	Retromer complex component A	VPS26A
Q96QK1	Retromer complex component	VPS35
O95782	Adaptor related protein complex 2 alpha 1 subunit	AP2A1
Q92538	Golgi brefeldin A resistant guanine nucleotide exchange factor 1	GBF1
A0A024R5S9	E3 ubiquitin protein ligase	NEDD4

3.2. Downregulated pathways.

Figure 5 shows the downregulated pathways with the corresponding number and percentage of proteins upregulated in EP+GF treatment. These differentially expressed proteins provide insight into the difference between the outcomes of GF-only treatments vs. EP+GF treatments.

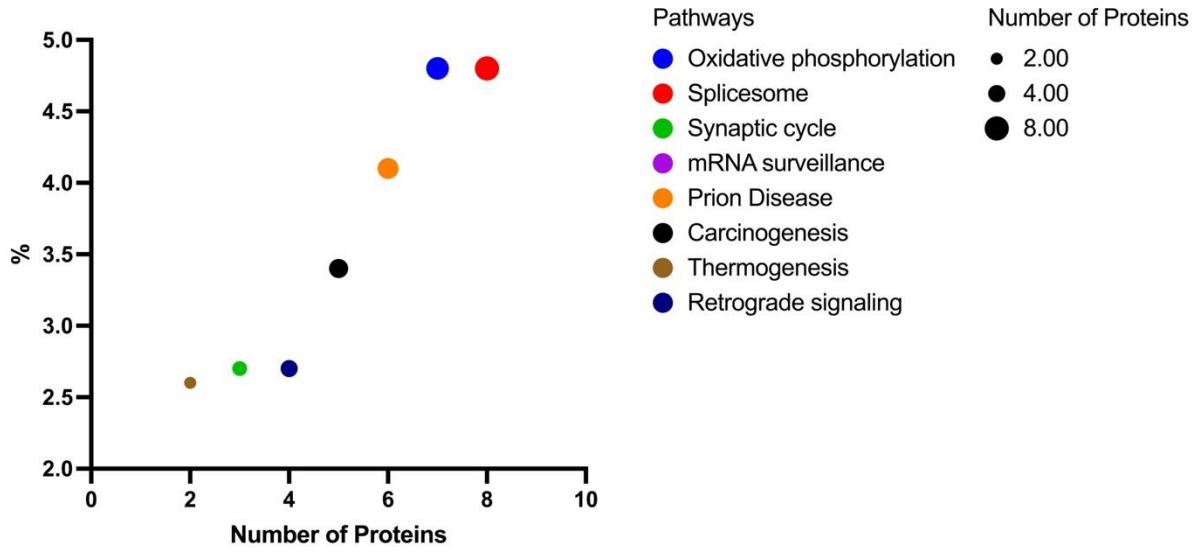

Figure 5. Downregulated pathways with the corresponding number and percentage of proteins upregulated in EP+GF treatment.

Spliceosomes are responsible and play a vital part in tumorigenesis. Spliceosome-induced splicing downregulation was observed in EP+GF treatment. CWC15 spliceosome associated protein homolog, LSM5 homolog, U6 small nuclear RNA and mRNA degradation associated, RNA binding motif protein 17, U2 snRNP associated SURP domain-containing (U2SURP), nuclear cap-binding protein subunit 2 (NCBP2), small nuclear ribonucleoprotein D2 polypeptide (SNRPD2), and survival motor neuron domain containing 1 are the key proteins that are significantly downregulated (SMNDC1). Along with spliceosome, ubiquitin-mediated proteolysis pathways are downregulated. They include Cbl proto-oncogene (CBL), cell division cycle 34 (CDC34), cullin 4A (CUL4A), and kelch-like ECH-associated protein 1 (KEAP1). The inhibition of the ubiquitin-proteosome pathway effectively halts the cell cycle. Downregulated proteins and genes in the Spliceosome pathway are displayed in Table 5.

Table 5. Downregulated proteins and genes in the Spliceosome pathway.

Protein ID	Gene Name	Gene ID
E9PB61	Aly/REF export factor	ALYREF
Q6I9S2	SNW domain containing 1	SNW1
Q8NI27	THO complex 2	THOC2

Protein ID	Gene Name	Gene ID
A0A024RAZ7	Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1
A0A023T6R1	Exon junction complex core component	MAGOHB
Q49AN9	Small nuclear ribonucleoprotein polypeptide G	SNRPG
O43290	Squamous cell carcinoma antigen recognized by T-cells 1	SART1
E9PB61	REF export factor	ALYREF
Q6I9S2	SNW domain containing 1	SNW1

Downregulation of key glycolytic genes LDHB and ENO1 were seen in Figure 6, along with a metabolic shift to mitochondrial ribosome upregulation, specifically in GF+EP treatment. Oxidative Phosphorylation was seen to be significantly upregulated, which suggests that the downregulation of glycolysis paves the way for alternative energy production pathways. This elevated OXPHOS could generate ROS to trigger a mitochondrion-mediated intrinsic apoptotic pathway of cell death.

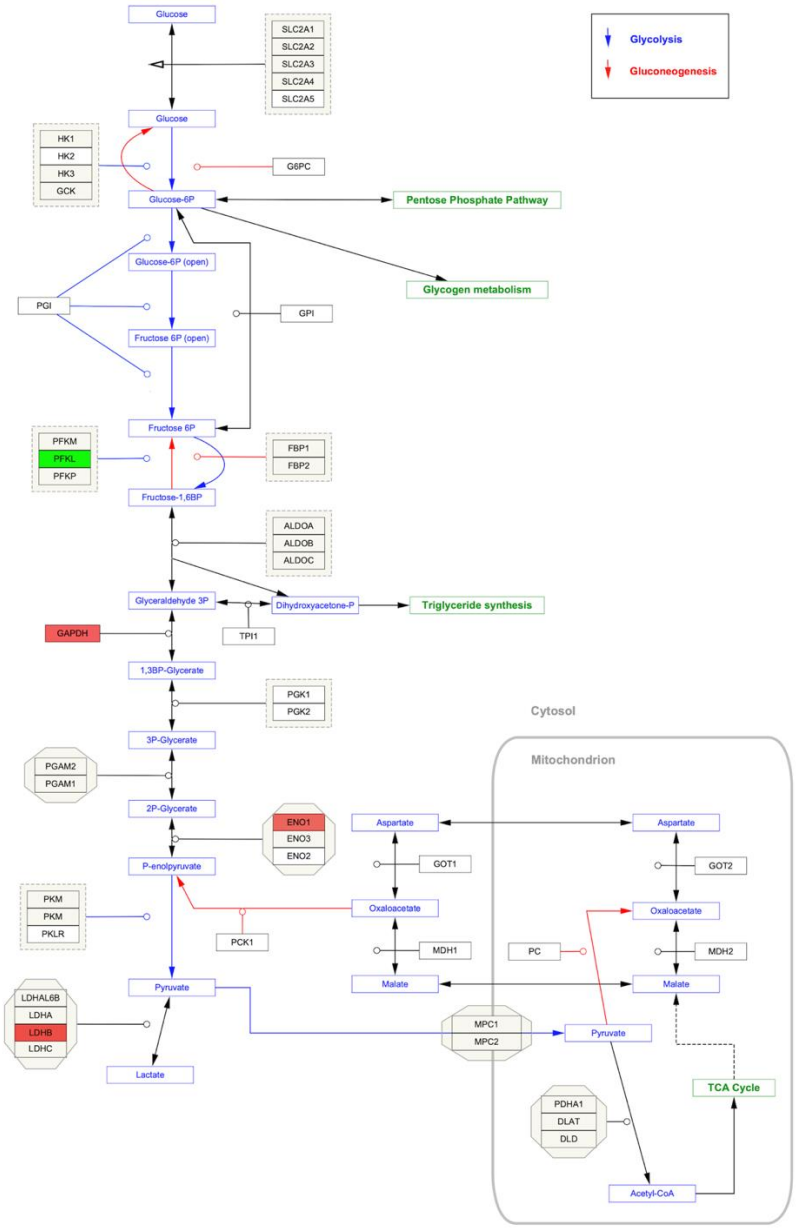

Figure 6. Identifying key glycolysis pathway proteins downregulated (red) and upregulated (green) in EP+GF compared to GF in MDA-MB-231 cells. Proteins from GO term "glycolysis/gluconeogenesis" from KEGG pathway analysis were uploaded to the Cytoscape 3.6.1 software and matched to the glycolysis pathway using the WikiPathway app (beta), with the degree of shading representing the fold change.

3.3. Gene ontology in biological process, cellular component, and molecular function.

Gene ontology (GO) analysis of significantly upregulated proteins was done to find the various proteins and their numbers involved in a given Biological Process, Cellular Component, or Molecular Function. The Upregulated genes in EP+GF treatments were identified and categorized and are shown in Figure 7. The highest number of genes were found localized in the membrane (85), cytosol (80), and cytoplasm (79) cell components. The 186 in protein binding and the 35 in RNA binding molecular functions indicate the significance of electroporation and upregulation of the effects of Galloflavin due to electroporation. This also supports prior results and data of Biological Processes, with 25 in protein transport and 19 in intracellular protein transport under biological processes.

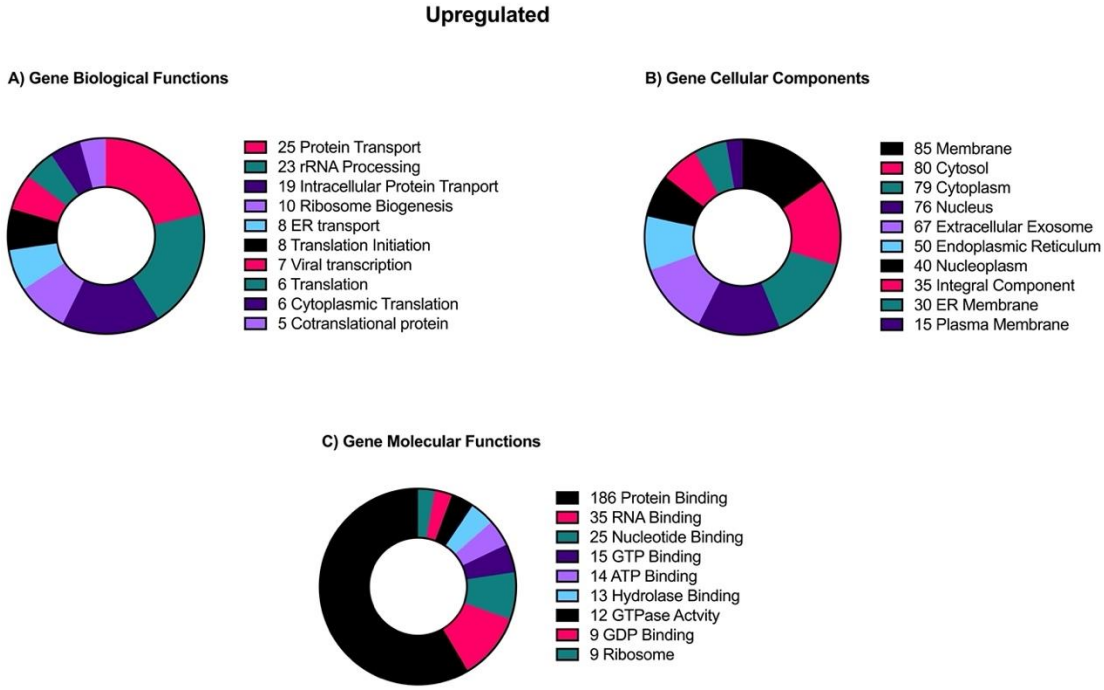

Figure 7. Gene ontology analysis of upregulated proteins.

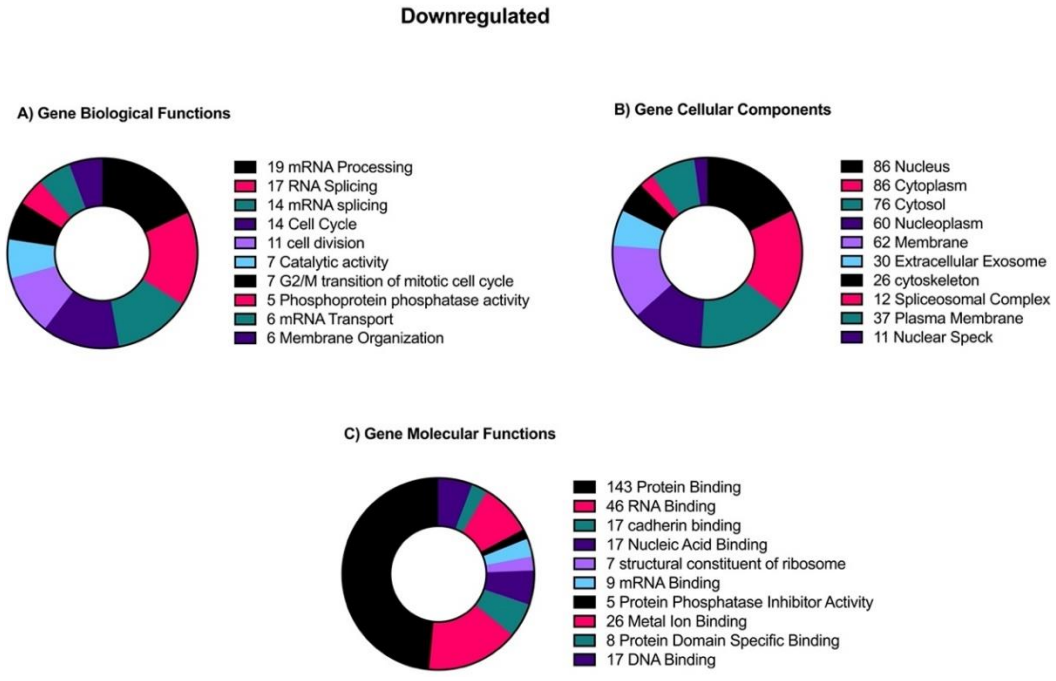

Figure 8. Gene Ontology analysis of Downregulated proteins.

The downregulated genes in EP+GF treatments were studied and categorized into Biological Processes, Cellular Components, and Molecular Functions. Figure 8 illustrates the details. Under biological processes, the highest number of genes were found in mRNA processing (19), 17 in RNA splicing, and 14 in mRNA splicing. Cell Cycle and Cell Division were also significantly downregulated with 14 and 11 genes in each, respectively. The molecular functions include 143 genes under protein binding, with 46 in RNA binding. Cellular components include localizations of 86 in the nucleus, 86 in the cytoplasm, 76 in the cytosol, and 60 and 62, respectively, in the nucleoplasm and membrane. All these support and provide evidence to our findings in the downregulated Spliceosome pathway and the downregulation of cell cycle and cell division is pro-apoptotic.

The above results were corroborated by the viability study (Figure 9) for 12h to 72h. A significant viability drop (80%) was seen in a combination of EP+GF with respect to control (normalized to 100%), indicating the efficacy of electrical pulse treatment using GF on MDA-MB-231 cells.

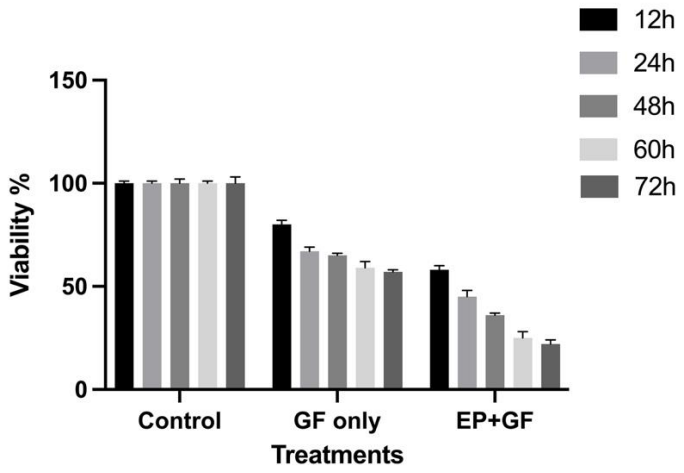

Figure 9. Viability of MDA-MB-231 cells at EP+GF compared with GF only and control treatments.

4. Conclusions

The proteomics study provides a significant landscape of molecular insights into what might be responsible for this treatment combination's anticancer effects. Proteomics analysis of EP+GF vs. GF only treatments identified a total of 3233 proteins, of which 2466 were common to both treatments, while 172 were significantly downregulated and 222 were significantly upregulated.

The various pathways identified that EP+GF act as antiproliferative and pro-apoptotic. In addition, Western Blotting done in previous studies validates the proteomics analysis by quantifying the downregulation of LDHB.

We can infer from the findings of proteomics investigations that the combined action of EP+GF activates numerous key proteins and pathways that eventually lead to a cellular apoptotic endpoint, as observed in the viability study. Apoptosis is initiated by suppressing proteins and genes, including LDHB, which downregulated the glycolysis pathway, which provides the major energy source for TNBC proliferation, supporting our findings. These key findings provide essential mechanistic insights into how EP+GF works as an antiproliferative therapy for TNBC MDA-231 cells. More research, particularly preclinical investigations, will help to translate the combinational treatment's potential value to clinical practice.

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Conflicts of Interest

The authors declare no conflict of interest.

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