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Development and Characterization of an Active Pharmaceutical Ingredient Using Polymeric Carriers Delivery System

Mohamed S. Abou-Taleb[1*], Mohamed A. Khattab[1], Ahmed A. Ma'amoun[1]

[1] Department of Materials Science, Institute of Graduate Studies and Research, Alexandria University, Egypt.

***Corresponding Author:**
Mohamed S. Abou-Taleb
Department of Materials Science, Institute of Graduate Studies and Research,
Alexandria University,
Egypt.
Email: Mohamed.selim12@alexu.edu.eg
mselim_research@yahoo.com
Tel: +201222707520

Abstract

Cardiac diseases are the major cause of mortality, morbidity, and disability. People are dying due to cardiac problems which include atherosclerosis, myocardial infarction and ischemic heart disease. Nanotechnology definition is the science and engineering involving the design, synthesis, characterization and application of materials and devices in at least one dimension is on the nanometer scale (one-billionth of a meter). Angiogenesis involving primary vascular plexus formation, which involves differentiation of endothelial cells from in situ mesoderm-derived precursor cells by sprouting or non-sprouting process. Gene therapy makes modification of gene expression for therapeutic gain, where a "normal" gene is inserted into the genome for replacement of an "abnormal" disease-causing gene. A carrier (a vector) is used for delivery of the therapeutic gene to the target cells of patient. Naked DNA – based gene transfer is to include in DNA solution substances capable of enhancing the efficiency of DNA internalization by target cells. Gene delivery involves the ability of DNA for crossing the cell membrane, escaping from endosome, and entering the nucleus because of the DNA's size and its negative charge. Vascular endothelial growth factor (VEGF) is considered as a potent endothelial cell mitogen in vitro, and a potent permeability mediator and angiogenic growth factor in

vivo. VEGF plays an important role for hypoxia-related angiogenesis occurring during wound healing, proliferative retinopathy, revascularization of ischemic areas. Poly (lactide-co-glycolide) (PLGA) is the most commonly used polymer for pharmaceutical controlled release devices. Polyester, PLGA is biodegradable and biocompatible, hydrolyzing to lactic and glycolic acid monomers. PLGA nanoparticles are potential vector for gene delivery. The comparison between fold expression in different treatments after 3, 5 and 7 days in relation to normal healthy and myocardial infarcted animals showed that there was statistical significant difference between normal healthy and other groups (P1 < 0.05) except PLGA-NPs – BSA treatment, between myocardial infarcted and other groups (P2 < 0.05), between PcDNA3.1-VEGF with treatment PLGA-NPs– BSA and PcDNA3.1-VEGF-PLGA-NPs Complex (P3<0.05) and between PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex (P4< 0.05). The effect of duration of treatment on each treatment type indicated that after 3, 5 and 7 days expression of PcDNA3.1-VEGF-PLGA-NPs Complex was higher than PcDNA3.1-VEGF and PLGA-NPs – BSA. There was statistical significant difference between 3 days and 5 days (P1 < 0.05), between 3 days and 7 days (P2< 0.05) and between 5 days and 7 days (P3<0.05).

Keywords: Cardiac diseases, Angiogenesis, Gene therapy, Naked DNA, Vascular Endothelial Growth Factor, Poly-Lactide-co-Glycolide.

1. Introduction

Cardiac diseases are the major cause of mortality, morbidity, and disability. Where people are dying of many cardiac problems which are atherosclerosis, myocardial infarction, arrhythmias, and ischemic heart disease.[1] Oral and systemic administration of drugs, though effective, does not provide appropriate therapeutic drug levels in the target arteries for sufficient periods of time.[2] Currently nanotechnology offering a broad platform in cardiovascular science field offering tools for exploring the frontiers of cardiac science at cellular level. Nanotechnology-based tools are used in treatment of the cardiovascular diseases. These are used in the areas of diagnosis, imaging, and tissue engineering. [2]

Cardiovascular diseases are the leading causes of death worldwide. Eighty million adults (one in three) in United States are estimated to have one or more types of cardiovascular disease. [3] CAD accounted for approximately 17.8 million deaths in 2017. The number is expected to grow to more than 22.2 million by 2030, [4] of these individuals, approximately 40% finally develop considerable congestive heart failure. Peripheral arterial disease (PAD) also affects approximately 8 million people in United States and is associated with significant mortality. [5]

Thus, nanotechnology is an effective treatment modality for achievement of localized and sustained arterial and cardiac drug therapy to prevent cardiovascular diseases. [6]

Nanotechnology is the science and engineering involving the design, synthesis, and characterization of materials whose smallest functional organization in at least one dimension is on the nanometer scale (one-billionth of a meter). [7, 8] The prefix "nano" is derived from the Greek word for "dwarf". Where one nanometer (nm) equals to one-billionth of a meter, or about 6 carbon atoms width or 10 water molecules. [9]

Gene therapy is an important method for genetic disorders treatment or prevention by correction of defective genes which are responsible for development of disease by delivering of repaired genes or the replacement of incorrect ones.[10] This takes place by the insertion of a normal gene into a certain location in the genome for replacement of non-functional defective gene. Swapping of an abnormal gene for a normal gene through homologous recombination or repairing through selective reverse mutation takes place, which helps in returning the gene to its normal function. [11] Nanotechnological applications tools in human gene therapy involving non-viral vectors based on nanoparticles (usually 50-500 nm in size) which can already be tested for transporting plasmid DNA. Nanotechnology would be applied in gene therapy for replacing currently used viral vectors by less immunogenic nanosized gene carriers. So, repaired genes delivery or replacing the incorrect genes are fields introduced greatly in nanoscale objects. [12]

Nanoparticles are colloidal particles having a size of 10 to 100 nm.[13] Nanoparticles and microparticles formulated using PLGA polymer are investigated as a non-viral gene delivery system due to they possess characteristics of sustained release, and are biocompatible, biodegradable, and are able for protecting DNA from endolysosomal degradation.[14]

The high efficiency of transfection occurs as the smaller fraction was not related to the differences in the DNA loading, cellular uptake, or the release of DNA from the two fractions. These results suggest that small particle size and uniform size distribution enhances gene expression mediated by nanoparticles. [15]

Nanoparticles formulation by PLGA polymer has greater gene transfection demonstration than formulation using PLA polymer in breast cancer this is due to higher DNA release from PLGA nanoparticles. PLGA has higher molecular weight resulted in forming nanoparticles with higher loading of DNA, demonstrating higher gene expression than those with formulation by low molecular weight PLGA. [16]

Nanotechnology offers a broad platform in the field of cardiovascular science by offering tools to explore the frontiers of cardiac science at the cellular level. Nanotechnology-based tools can be effectively used to treat the cardiovascular diseases. [17]

Angiogenesis is considered as a complex process in which its regulation is done by stimulatory and inhibitory factors and its initiation occurs when there is a predominance of angiogenic factors that favour the growth of new vessel (e.g. Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), and transforming growth factors alpha and beta), which are called 'angiogenic switch' [18] VEGF is a potent regulator of angiogenesis, both physiologically and pathophysiologically. It is a potent mitogen, a survival factor for endothelial cells, and also mediates vessel permeability and migration of bone marrow, endothelial progenitor cell. [19]

Angiogenesis is the key process involved in normal development and wound repair and is motivated by different kinds of growth factors for instance vascular endothelial growth factor (VEGF) which is crucial angiogenic growth factor by increasing proteins and particulates or permeability vascular leak. The main function of VEGF is the production of new blood vessels. In adults they are responsible for recovery of vascular injuries, this occurs by new vessels creation for evading blocked vessels. [20]

Non-viral gene transfer has been used for delivering genes to ischemic tissues for angiogenic therapy. Genes encoding proteins stimulating angiogenic signal transduction are administered by cationic polymers, lipids, liposomes and three-dimensional (3D) scaffolds. [21]

Angiogenic therapy, induction occurs by administration of exogenous genes which encode angiogenic growth factors, are used as a potential treatment for patient that can't be treated with surgical intervention or angioplasty. [22] Gene transfering of angiogenic growth factors includes vascular endothelial growth factor (VEGF), [23] basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) which enhance angiogenesis and recover ischemic tissues in an animal model of myocardial infarction. [24]

Gene therapy has modification of gene expression for therapeutic gain; it is dependent on an understanding of basic biological principles. In many, a "normal" gene insertion occurs into the genome of diseased patient to replace an "abnormal" gene. A carrier vector is used for delivering therapeutic gene by targeting to the patient's cells. Common vectors are viruses that are genetically altered for carrying normal human DNA, but they attack their hosts by introducing their genetic material as a part of their replication cycle into the cell of host, and therefore they are good vehicles for carrying good genes into the cell of human. Many effects are undesired due to toxicity of vectors or

their immunogenicity, and the by viruses the target cells in the body will be infected, and the inserted gene must not disrupt any vital genes already in the genome. [25]

Despite the high delivery efficiency of viral vectors, there are still no commercial products have an approval from Food and Drug Administration. [26] Therefore; attention has become focused on the developing of vectors for non-viral gene delivery. Non-viral approaches began with simply delivering naked plasmid DNA. Non-viral delivery has employed synthetic chemical vectors to deliver the genes of interest. Synthetic chemical vectors composed of biodegradable materials could avoid the safety issues of viral vectors which are easy in manufacturing, purifying, well chemically modification, and scaling-up more than that of viral vectors. [27]

Many synthetic materials are used as non-viral vectors which include cationic polymers, cationic lipids, liposomes, inorganic nanoparticles, and dendrimers. Such materials make binding to, complex with, or encapsulating DNA. [28]

Naked DNA, which is in the plasmid form, is considered as the simplest form of non-viral transfer of the gene into a target cells. Non-viral delivery vectors are classified as organic (lipid complexes, conjugated polymers and cationic polymers) and also, inorganic (magnetic nanoparticles, quantum dots, carbon nanotubes and gold nanoparticles) systems. [29]

Non-viral gene carriers contain primarily of a cationic region to condense the anionic therapeutic plasmid, thus protecting the plasmid from degradative nucleases. Non-viral vectors used possess low toxicicity and immunogenicity compared to viral vectors. High - profile of non-viral vectors due to the safety offered by these systems when compared with viruses. [30]

Non-viral delivery of genes is safe than its viral delivery counterpart. Strategies were done to enhance non-viral gene delivery involving complexing plasmids with cationic polymers or lipids that self-assemble with DNA forming particles that are able for cells endocytosis. [31]

Polymer-based gene vector that has been successfully used in intravascular gene therapy utilizes the biocompatible and biodegradable copolymer poly-D-L-lactide-co-glycolide (PLGA), which a polymer of few synthetic ones that have been approved for use in humans. [32]

The advantages of the chemically based, non-viral systems are numerous, on stems. Besides their lesser toxicity and the lower immune responses to the non-viral vector, no integration into the genome occurs. [33]

Plasmid DNA is considered as the large negatively charged molecule up to 1μm in length and the cell membrane is an equally negatively charged surface. As cell membrane has been crossed by

endocytosis, releasing of DNA takes place from the endosomal compartment before degradation is done by the endosomal enzymes and then reaching of nucleus. [34]

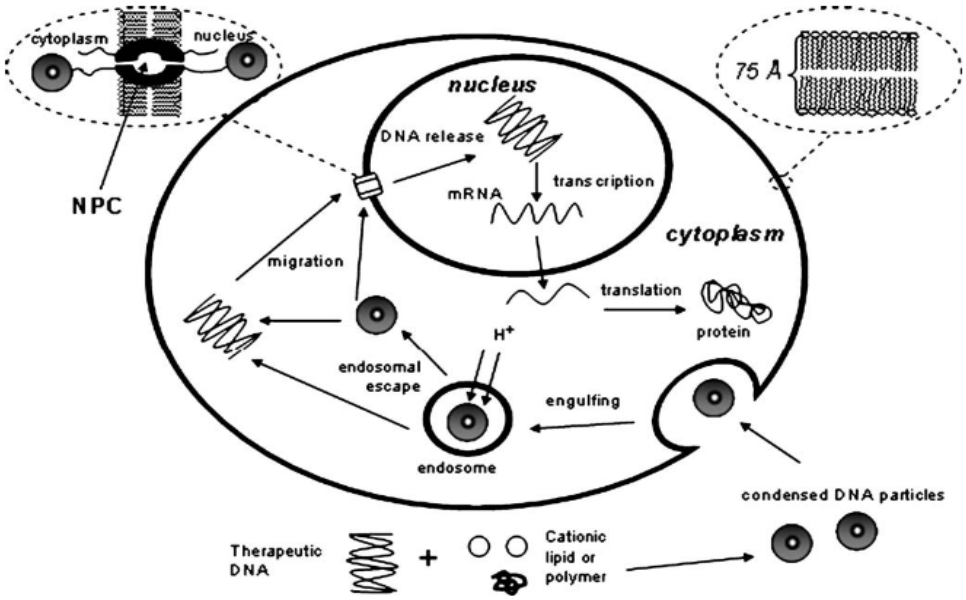


Figure 1: Non-viral gene therapy.

Gene delivery systems using Polycation incorporates the complexes an active motif enhancing membrane permeability, a nuclear localization signal for enhancement nuclear import, a targeting ligand process to membrane receptors for increasing affinity to binding, and/or compounds to facilitate DNA dissociation from the complexes when inside the cytoplasm. Cationic polymers and other naturally occurring compounds have proven to be extremely effective for in vitro gene delivery. While effective in protecting DNA from DNA degradation, the polycations in either lipoplexes or polyplexes have the intrinsic property of causing significant aggregation in biological matrices full of negatively charged molecules, and of preventing effective release of DNA once inside the cells. [35]

A gene transfer agent (GTA) has to fulfill several functions, including: packaging of pDNA, secure and efficient delivery of the transgene into the target cells and their nuclei, providing protection from DNA degradation, and ensure site-specific controlled gene expression in the cell. [35]

The VEGF family has led to the discovery of several members encoded by different genes: VEGF-A of vascular permeability factor (firstly discovered), B, C, D, E, F and PDGF (Platelet Derived Growth Factor). The most widely studied family member as far as angiogenesis is concerned has been VEGF-A, which we will refer to as VEGF from now on. Different VEGF isoforms form alternative splicing are: VEGF121, 145, 165, 189 and 206 (number is an indication of amino acid residues for VEGF). VEGF165 is called soluble heparin binding protein, and is less in diffusion than VEGF121, but it has higher in mitogenic activity. [36]

Vascular endothelial growth factor (VEGF) is considered as potent endothelial cell mitogen in vitro [37] and a potent permeability mediator [38] and angiogenic growth factor in vivo. [39] VEGF plays an important role in hypoxia-related angiogenesis which occurs during wound healing process [40], proliferative retinopathy [41] revascularizing of ischemic areas [42] and tumor progression, [43] within the vascular wall, VEGF can be produced using vascular smooth muscle cells (VSMCs) [44] and can thereby modulate endothelial cell function via a paracrine pathway. VEGF has been shown that playing an important role in the process of atherogenesis [45] and neovascularization in atherosclerotic plaque. [46]

Increased VEGF secretion and expression induced by cyclical stretching is relevant to cardiovascular system pathologic states, this includes atherosclerosis and hypertension. Cyclical stretching-induced increases in VEGF production, plays an important role in vascular injury that complicates disease states such as diabetes mellitus. [47]

Poly (lactide-co-glycolide) (PLGA) is the most commonly used polymer for pharmaceutical controlled release devices. [48] The polyester, PLGA is biodegradable and biocompatible, hydrolyzing to monomers of lactic and glycolic acid. Hydrolysis of PLGA is considered as an invariant description of a solid formulation, which includes the glass transition temperature (Tg), moisture content and polymer molecular weight. [49] PLGA properties changes during polymer biodegradation influencing rates release and degradation of the incorporating molecules of drug. [50] Physical properties of PLGA shows to be depending on many factors, which includes the initial molecular weight, ratio of lactide to glycolide monomers, device size, water exposure (both reactant and plasticizer), and temperature of storage. [51]

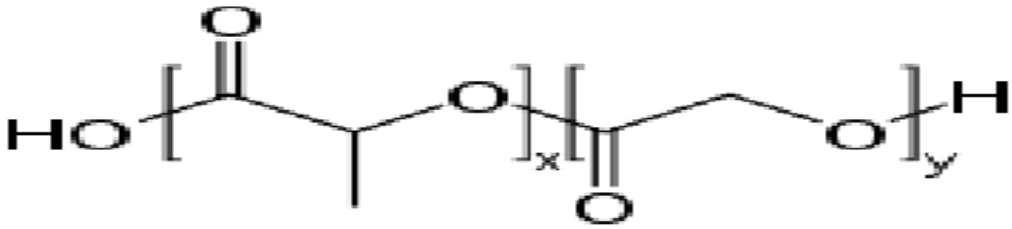


Figure 2: Structure of Poly (D, L-lactide-co-glycolide) (PLGA).

Poly- lactic-co-glycolic acid (PLGA) shows a potential effect as a drug delivery carrier and as scaffolds for tissue engineering. PLGA polymer is FDA-approved as a biodegradable polymer, which is physically strong and has high biocompatiblity and is used as delivery vehicles for drugs, proteins and macromolecules like DNA, RNA and peptides. PLGA is showing that it is the most biodegradable polymer due to its favorable degradation characteristics and possiblity for sustained drug delivery process. [52]

Degradation of PLGA copolymer takes place by hydrolysis or biodegradation by cleavage of its backbone ester linkages into oligomers then to monomers. Carboxylic end groups increase resulting from biodegradation process of auto-catalyses. PLGA copolymer degradation is a collective process of bulk diffusion, surface diffusion, bulk erosion and surface erosion processes. [53]

The biodegradation rate of the PLGA copolymers are dependent on the molar ratio of the lactic and glycolic acids in the polymer chain, molecular weight of the polymer, the degree of crystallinity, and the Tg of the polymer. The release of drug from the homogeneously degrading matrix is more complicated. [54]

2. Aim of the work

To detect the therapeutic effect of PLGA – Nanoparticles after gene transfection using PcDNA 3.1 carrying VEGF targeted to myocytes in myocardial infarcted animal model. Also, to evaluate the therapeutic effect of PcDNA3.1-VEGF –PLGA – NPs (gene transfer complex, polyplex), the effect of therapeutic material PcDNA 3.1-VEGF and also PLGA – NPs – BSA for myocardial infarcted animals treatment.

3. Materials and Methods

3.1 Materials:

We used 20 male rats: 4 healthy rats were used as negative control, and blood samples were collected and 16 animals had induced myocardial infarction with Isoprenaline Hydrochloride (5 g, MW 247.72 g /mol), 4 animals were used as positive control and blood samples were collected after one day of injection then animals were sacrificed (terminated) , and 12 rats were used as treatments had induced myocardial infarction, then were divided into three treatment groups (4 rats each) to be injected with therapeutic materials.

-Group I (Negative control): Non – injected 4 normal healthy animals which were used as negative control, blood samples were collected immediately, animals were sacrificed (terminated) and hearts were taken.

-Group II (positive control): 4 normal healthy animals were induced myocardial infarction and left a day then blood samples were collected from each animal and animals were sacrificed and hearts were taken.

-Group III (Treatments): 12 animals, had induced myocardial infarction, which were used as treatments and were divided into three treatment groups:

a- Treatment 1: Injected to induce myocardial infarction, then animals were injected with PcDNA 3.1 carrying VEGF (Therapeutic gene), which was used as treatment 1 (4 animals), blood samples were collected, animals were sacrificed and hearts were taken.

b- Treatment 2: Injected to induce MI then animals were injected with PLGA – NPs -BSA which was used as treatment 2 (4 animals), blood samples were collected, animals were sacrificed and hearts were taken.

c- Treatment 3: Injected to induce MI, then animals were injected with PcDNA3.1 carrying VEGF coupled with PLGA – NPs which is carrier polymer (PLGA MW 50000g / mol), forming a gene transfer complex (polyplex) which was used as treatment 3 (4 animals), blood samples were collected, animals were sacrificed and hearts were taken. A day was left (24 hours) to induce MI in rats after injection with Isoprenaline HCl for positive control group and for treatments group to begin treatment course.

A blood sample from rats in each group was taken immediately from group I (Healthy animals) immediately, from infarcted animals after one day, and after three, five, and seven days of infarction from treatments group into EDTA tubes to make Q-real time PCR. Also a blood sample was taken from all animal groups in plain tubes to make cardiac profile.

After treatment took place animals were sacrificed (terminated) and hearts were taken from healthy, infarcted and treated animals and put in 10% ne formaldehyde, dehydrated with alcohol 70-100%, cleared in Xylene, impregnated in molten paraffin to make paraffin blocks, the blocks were cut into sections using microtome, then sections were deparaffinized with xylene and rinsed in haematoxylin and eosin stain, then washed and dehydrated in alcohol and cleared in xylene. The slides were mounted by DPX and covered to be investigated under light microscope.

3.2. Methods:

The groups of animals Non-injected, injected to induce MI using (Isoprenaline Hydrochloride) and the three treatment groups were listed in the following table:

Groups / Injected	Group I (-ve control Normal healthy)	Group II (+ve control, Infarcted Animals)	Group III (Treatments)			No. of Rats
			T1	T2	T3	

No injection	**Non**	**--**	**--**	**--**	**--**	**4 animals**
Injection with Isoprenaline-HCL	**--**	**Injected**	**Injected**	**Injected**	**Injected**	**16 animals**
Injection with PcDNA3.1 carrying VEGF alone	**--**	**--**	**Injected**	**--**	**--**	**4 animals**
Injection with PLGA-NPs –BSA	**--**	**--**	**--**	**Injected**	**--**	**4 animals**
Injection with PcDNA 3.1-VEGF -PLGA -NPs complex	**--**	**--**	**--**	**--**	**Injected**	**4 animals**

Table (1): Groups, Materials injected and number of animals

3.2.1 The PcDNA3.1- VEGF construct:

a- Many Plasmids were used as expression vector which can be propagated in Ecoli, pcDNA3.1, was purchased from Invitrogen carrying VEGF.

b- The cDNA fragment of human VEGF cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR), using template mRNA, and a set of primers for VEGF were (forward: 5'-TGC ACC CAC GAC AGA AGG A -3'; its Molecular weight was 5816.8 ug/umole and reverse: 5'-GGC AGT AGC TTC GCT GGT AGA C -3', its Molecular weight was 6792.4 ug/umole). Also, B-actin primers sequences (Forward: 5'- GGC TGT ATT CCC CTC CAT CG - 3'its Molecular weight was 6021 ug/umole and Reverse: 5'- CCA GTT GGT AAC AAT GCC ATG T - 3', its Molecular weight was 6735.4 ug/umole), which were used as reference gene sequences.

c- The cDNA sequence was confirmed and the EcoRI/HindIII fragment of the cDNA was subcloned into the EcoRI/HindIII sites of the PcDNA3.1, resulting in PcDNA3.1- VEGF, the PcDNA3.1-VEGF concentration was 372.62 ug / ml.

Many *E. coli* strains are suitable for the propagation of this vector including, DH5-alpha, the propagating vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A) which were more recommended.

3.2.2 Transformation Method for PcDNA3.1 carrying VEGF in E.coli: [55]

1- To propagate and maintain PcDNA3.1, 10 ng of vector carrying VEGF gene was used to transform a *rec*A, *end*A *E. coli* strain DH5-alpha.

2- Transformants were selected on LB plates containing 50–100 μg/ml ampicillin.

3- A glycerol stock was prepared of the plasmid-containing E. *coli* strain for long-term storage.

4- PcDNA3.1 was a non-fusion vector. The insert should contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation. ATG initiation codon is (G/A) NNATGG.

5- The insert also contained a stop codon for proper termination of the gene.
6- The *Xba* I site contained an internal stop codon (TCTAGA).

7- Below was the multiple cloning site for PcDNA3.1. Restriction sites were labeled in the genetic map to indicate the cleavage site. The *Xba* I site contained an internal stop codon (TCTAGA).

8- E. coli Transformation:

Once you had obtained a clone containing the gene of interest, you might, transforming the clone into a suitable *E. coli* host occured.

9- Preparation of Glycerol Stock:

Once you had identified the correct clone, purify the colony and made a glycerol stock for long-term storage. A DNA stock of plasmid at –20°C should be kept.

10- The original colony was streaked out on an LB plate containing 50 μg / ml ampicillin.
11-The plate was incubated at 37°C overnight.
12- A single colony was isolated and inoculated into 1–2 ml of LB containing 50μg/ml ampicillin.
13- The culture was grown to mid-log phase (OD600 = 0.5–0.7).
14- 0.85 ml of culture was mixed with 0.15 ml of sterile glycerol and transfer to a cryovial. Store at –80°C.

15-Transfection:

Once it had verified that the gene was cloned in the correct orientation and contained an initiation ATG and a stop codon, you were ready to transfect the construct to host.

16-Plasmid Preparation:

Once we had generated our expression clone, we might isolate plasmid DNA carrying gene for transfection. Plasmid DNA for transfection into eukaryotic cells must be cleaned and free contamination with from phenol and sodium chloride. Contaminants would kill the cells, and salt would interfere with lipid complexing, decreasing transfection efficiency.

3.2.2.1. PcDNA3.1-VEGF description and genetic map: [56]

The synthetic gene PcDNA3.1 carrying VEGF was assembled from synthetic oligonucleotides and/or PCR products. The fragment was inserted into PcDNA3.1 (+). The plasmid DNA was purified from transformed bacteria and Concentration determined by UV spectroscopy. The sequence congruence within the insertion sites was 100%. Lyophilized 5 μg of the plasmid carrying gene was constructed.

The following Figure showed the Genetic map of the construct, PcDNA3.1-VEGF:

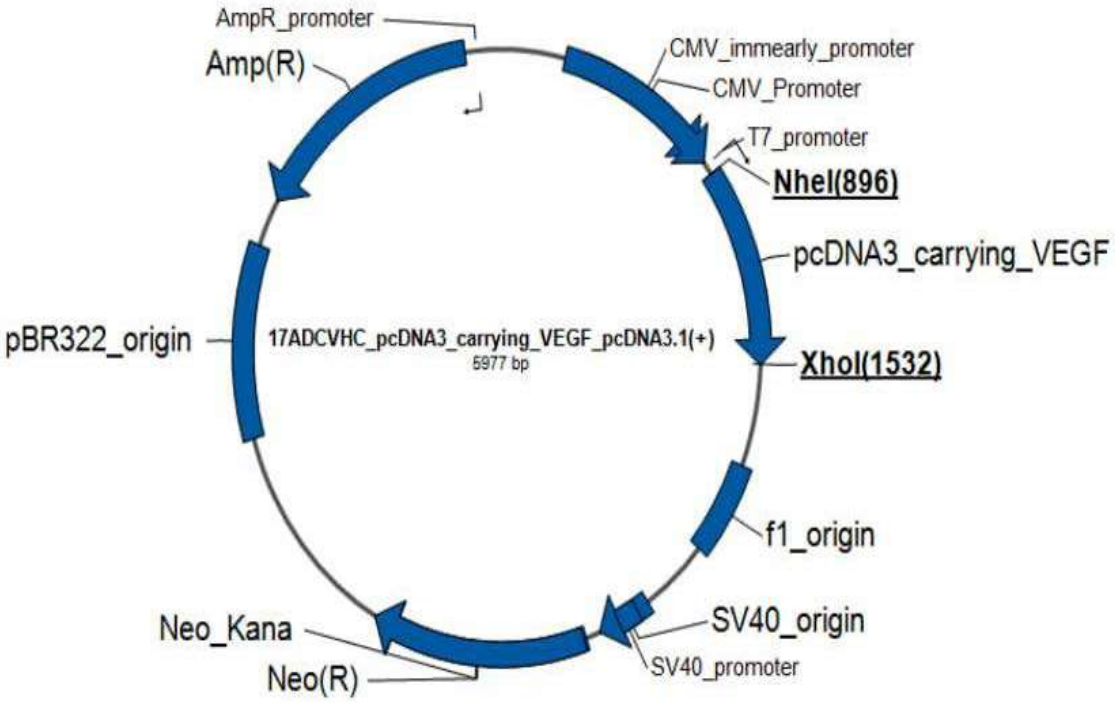


Figure 3: Genetic Map for PcDNA3.1 (+) carrying VEGF.

3.2.2.2. Preparation of competent cells:

a) E.coli was grown in 50 ml LB for overnight at 37 ^{0}C, 150 rpm.

b) 1ml of pre- grown was inoculated overnight culture to 100 ml LB broth (10g/l) trypton, 5 g/l yeast and 5g/l NaCl.

c) Incubation at 37 ^{0}C for 2-3 hrs until absorbance at 600 nm or 420 nm is 0.2-0.4; and centrifugation at 200 rpm took place.

d) The growth was stopped on ice for 30 min and collected on falcon tubes 15 ml.

e) Cell harvesting at 5000 rpm, 4 ^{0}C for 20 minutes occurred.

f) Decanting supernatant took place; cells were suspended in 10 ml pre-cooled at 0.1 M $CaCl_2$ and collect in two falcons.

g) Falcons were kept on ice for 30 min.

h) Cell harvesting by centrifugation at 4000 rpm, 4 ^{0}C for 20 min.

i) Decanting supernatant, suspension of cells in 1-2 ml pre-cooled 0.1 M $CaCl_2$ was made.

j) Competent cells were kept at 4 ^{0}C for 24 hours.

3.2.2.3. Transformation of E.Coli:

a) 200 ml competent cells were added to 10 ml PGEM T recombinant for ligation and add 5 ul construct (PcDNA3.1-VEGF) in an epindorf.

b) Keeping on ice for 40 minutes was made.

c) Heat shock at 42 ^{0}C for 45 seconds took place.

d) Keeping on ice for 5 minutes was done.

e) 200 ml E.coli recombinant was added to 5 ml LB broth in 50 ml falcon tube.

f) Incubation at 37 ^{0}C for 2-4 hours , 200 rpm was made

g) Plating on LB/Amp/IPTG/x-gal agar plates took place.

h) Incubation at 37 ^{0}C overnight was made.

i) Inoculating 1ml E.coli recombinant in 100 ml LB broth and 100 ml Amp was done.

j) Selection of colonies was made.

k) Purifying PcDNA3.1-VEGF from colonies using Plasmid purification kit was applied.

3.2.3. Preparation and purification of PcDNA3.1 carrying VEGF [57]

The components used for preparation and purification of PcDNA3.1 carrying VEGF were:

a) Resuspension Solution, 15 mL.

b) Lysis Solution, 15 mL.

c) Neutralization Solution, 20 mL.

d) Wash Solution (concentrated), 20 mL.

e) Rnase A (10 mg/mL), 0.15 mL.

f) Elution Buffer (10 mM Tris-HCl, pH 8.5), 4 mL.

g) GeneJET Spin Columns, 50 columns.

e) Collection Tubes (2 mL), 50 tubes.

3.2.3.1. Principle:

1- Pelleted bacterial cells were resuspended and subjected to SDS/alkaline lysis to liberate the plasmid DNA.

2-The resulting lysate was neutralized to create appropriate conditions for binding of plasmid DNA on the silica membrane in the spin column.

3-Cell debris and SDS precipitate were pelleted by centrifugation, and the supernatant containing the plasmid DNA was loaded onto the spin column membrane.

4-The adsorbed DNA was washed to remove contaminants, and was then eluted with a small volume of the Elution Buffer (10 mM Tris-HCl, pH 8.5).

5-The purified plasmid DNA was ready for immediate use in all molecular biology procedures such as PCR, transformation and automated sequencing.

3.2.3.2. Buffer Preparation:

1- The provided RNase A was added solution to the Resuspension Solution and mixed. After addition of RNase A, the Resuspension Solution could be used for 6 months when stored at 4°C.

2- Ethanol (96-100%) was added to the Wash Solution prior to first use:

Wash Solution (concentrated)	20 mL
Ethanol 96%	35 mL
Total volume	55 mL

3- The Lysis Solution and the Neutralization Solution were checked for salt precipitation before being used. Any precipitate was re-dissolved by warming the solution at 37°C, and then was cooled back down to 25°C before being used. The Lysis Solution was shaken too vigorously.

3.2.3.3. Growth of Bacterial Cultures:

1- A single colony was picked from a freshly streaked selective plate to inoculate 1-5 mL of LB medium supplemented with the appropriate selection antibiotic and was incubated for 12-16 hours at 37°C while shaking at 2000-2500 rpm. A tube or flask was used with a volume of at least 4 times the culture volume.

2- The bacterial culture was harvested by centrifugation at 8000 rpm in a microcentrifuge for 2 min at room temperature. Decantion of the supernatant and removing all remaining medium occurred.

3- For high-copy-number plasmids, 5 mL of bacterial culture was taken.

4- For low-copy-number plasmids, larger volumes of bacterial culture (up to 10 mL) were taken to recover a sufficient quantity of DNA.

3.2.3.4. Plasmid DNA Purification Protocol:

1- All purification steps were carried out at room temperature.

2- All centrifugations was carried out in a table-top micro-centrifuge at 10 000 -14 000 rpm.

3- 1-5 mL of *E. coli* culture in LB media was used for purification of high- copy plasmids.

4- For low-copy plasmids, up to 10 mL of culture was used.

The Protocol steps of Plasmid PcDNA3.1-VEGF purification was as follows:

1- The pelleted cells were resuspended in 250 uL of the Resuspension Solution. The cell suspension was transferred to a microcentrifuge tube. The bacteria should had been resuspended completely by vortexing or pipetting up and down until no cell clumps remained (RNase A had been added to the Resuspension Solution).

2- 250 uL of the Lysis Solution was added and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear. Vortexing wasn't made to avoid shearing of chromosomal DNA. Incubation wasn't made for more than 5 min to avoid denaturation of supercoiled plasmid DNA.

3- 350 uL of the Neutralization Solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times. It was important to mix thoroughly and gently after the addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris.The neutralized bacterial lysate should became cloudy.

4- Centrifugation for 5 min to pellet cell debris and chromosomal DNA took place.

5- The supernatant was transferred to the supplied GeneJET spin column by decanting or pipetting. Disturbing or transferring the white precipitate was avoided. The bag with GeneJET Spin Columns was closed tightly after each use.

6- Centrifugation for 1 min took place. The flow-through was discarded and the column was placed back into the same collection tube. Bleach wasn't added to the flow-through.

7- The GeneJET spin column was washed by adding 500 uL of Wash Solution I (Diluted with isopropanol) and centrifugated for 30-60 sec. The flow-through was discarded, this step was essential to remove trace nuclease activity.

8- 500 uL of the Wash Solution was added (diluted with ethanol) to the GeneJET spin column. Centrifugation for 30-60 seconds took place and the flow-through was discarded. The column back was placed into the same collection tube.

9- The wash procedure was repeated using 500 uL of the Wash Solution.

10- The flow-through was discarded and centrifuged for an additional 1 min to remove residual Wash Solution. This step was essential to avoid residual ethanol in PcDNA3.1-VEGF preps.

11- The GeneJET spin column was transferred into a fresh 1.5 mL microcentrifuge tube. Then 50 uL of the Elution Buffer was added to the center of GeneJET spin column membrane and elution of the PcDNA3.1-VEGF took place. Incubation for 2 min at room temperature and centrifugation for 2 min occurred.

12- The column was discarded and the purified PcDNA3.1-VEGF was stored at -20°C.

3.2.4. Preparation and Purification of RNA : [58]

-The GeneJET RNA Purification Kit was a simple and efficient system for purification of total RNA from mammalian cultured cells, tissue, human blood cells, bacteria, yeast and insects.

-The kit utilized a silica-based membrane technology in the form of a convenient spin column, eliminating the need for tedious cesium chloride gradients, alcohol precipitation or toxic phenol chloroform extractions.

-RNA molecules longer than 200 nucleotides could be isolated with the GeneJET RNA Purification Kit in 15 minutes after the lysis step. The high-quality purified RNA could be used in a wide range of downstream applications including RT-PCR, RT-qPCR, Northern blotting and other RNA-based analyses.

3.2.4.1. Principle:

Samples were lysed and homogenized in Lysis Buffer, which contains guanidine thiocyanate, achaotropic salt capable of protecting RNA from endogeneous RNases .The lysate was then mixed with ethanol and loaded on a purification column. The chaotropic salt and ethanol caused RNA to bind to the silica membrane while the lysate is spun through the column.

Subsequently, impurities were effectively removed from the membrane by washing the column with wash buffers. Pure RNA was then eluted under low ionic strength conditions with nuclease-free water.

3.2.4.2. Blood Cells Total RNA synthesis and Purification Protocol:

3.2.4.2.1. Before starting:

1- Supplementing the required amount of Lysis Buffer with β-mercaptoethanol.

20 µL of 14.3 M β-mercaptoethanol was added to each 1 mL volume of Lysis Buffer required.

2- Blood sample collection from all rats and RNA purification from blood cells was carried out within the same day. Samples were stored at 4°C until use. Freezing of blood samples wasn't done.

3.2.4.2.2. Step Procedure:

1- Blood cells were collected by centrifugation of 0.5 mL of whole blood at 400 × g for 5 min at 4^{o}C. Blood cells would generate a pellet of approximately 60-70% of the total sample volume. Removing the clear supernatant (plasma) from the pellet with a pipette was made.

2- Resuspension of the pellet in 600 µL of Lysis Buffer supplemented with β-mercaptoethanol took place. Vortexing or pipette was made to mix thoroughly.

3- Adding 450 µL of ethanol (96-100%) and mixing by pipetting took place.

4- Transferring up to 700 µL of lysate to the GeneJET RNA Purification Column inserted in a collection tube was done. Centrifugation the column for 1 min at ≥12000 × g and discarding the flow through was made and the purification column back into the collection tube was placed.

5- Repeating this step until all of the lysate had been transferred into the column and centrifugation was made. Discarding the collection tube containing the flow-through solution took place. Placing the GeneJET RNA Purification Column into a new 2 mL collection tube was done.

6- 700 µL of Wash Buffer 1 (supplemented with ethanol) was added to the GeneJET RNA Purification Column and centrifugation for 1 min at ≥12000 × g took place. The flow through was discarded and placed the purification column back into the collection tube.

7- 600 µL of Wash Buffer 2 (supplemented with ethanol) was added to the GeneJET RNA Purification Column and centrifugation for 1 min at ≥12000 × g took place. The flow through was discarded and placed the purification column back into the collection tube.

8- 250 µL of Wash Buffer 2 was added to the GeneJET RNA Purification Column and centrifuged for 2 min at ≥12000 × g. If residual solution was seen in the purification column, the collection tube was empty and re-spinning the column for 1 min. at maximum speed was made.

9- Discarding the collection tube containing the flow-through solution and transfering the GeneJET RNA Purification Column to a sterile 1.5 mL RNase-free microcentrifuge tube was done.

10- 50 µL of Water, nuclease-free (included) was added to the center of the GeneJET RNA Purification Column membrane. Centrifugation for 1 min at ≥12000 × g to elute RNA was made.

11- Discarding the purification column occurred. The purified RNA was used for downstream applications or RNA was stored at -20°C or -70°C until use.

3.2.5. Treatment of RNA with DNase I: [59]

3.2.5.1. Description:

1-DNase I was an endonuclease that digested single- and double-stranded DNA. It hydrolyzed phosphor diester bonds producing mono- and oligo-deoxy-ribonucleotides with 5'-phosphate and 3'-OH groups.

2-The enzyme activity was strictly dependent on Ca^{2+} and is activated by Mg^{2+} or Mn^{2+} ions: In the presence of Mg^{2+}, DNase I cleaved each strand of dsDNA independently, in a statistically random fashion; In the presence of Mn^{2+}, the enzyme cleaved both DNA strands at approximately the same site, producing DNA fragments with blunt ends or with one or two nucleotide overhangs.

3.2.5.2. Applications of DNase I:

1- Preparation of DNA-free RNA.

2- Removal of template DNA following *in vitro* transcription.

3- Preparation of DNA-free RNA prior to RT-PCR and RT-qPCR.

4- DNA labeling by nick-translation in conjunction with DNA polymerase I.

5- Studying of DNA-protein interactions by DNase I, RNase-free footprinting.

6- Generation of a library of randomly overlapping DNA inserts. Reaction buffer containing Mn2+ is used.

Its Source was *E.coli* cells with a cloned gene encoding bovine DNase I, and Molecular Weight was 29 k Da monomer. One unit of the enzyme completely degraded 1 µg of plasmid DNA in 10 min at 37 °C. Inhibitors were: metal chelators, transition metals (e.g., Zn) in millimolar concentrations. Inactivation occurred by heating at 65 °C for 10 min in the presence of EDTA

3.2.5.3. Removal of genomic DNA from RNA preparations protocol:

1. The following was added to free tube:

RNA	1 µL
10X reaction buffer with MgCl2	1 µL
DNase I, RNase-free	1 µL (1 U)
DEPC-treated Water	to 10 µL

2. Incubation at 37 °C for 30 min was made.

3. 1 µL 50 mM EDTA was added and incubated at 65 °C for 10 min. RNA hydrolyzed during heating with divalent cations in the absence of a chelating agent. Alternatively, phenol/chloroform extraction was used.

4. The prepared RNA was used as a template for reverse transcriptase.

3.2.6. Synthesis of cDNA from total RNA: [60]

3.2.6.1. Components:

The kit included the following reagents:

1) 10× RT Buffer, 1.0 mL 1 tube.
2) 10× RT Random Primers, 1.0 mL 1 tube.
3) 25× dNTP Mix (100 mM), 1 tube, 0.2 mL.
4) Reverse Transcriptase, 50 U/µL, 2 tubes, 0.1 mL.
5) RNase Inhibitor, 100 µL, 2 tubes.

3.2.6.2. Protocol of cDNA synthesis:

3.2.6.2.1. Preparation of 2X RT master mix:

1) The kit components were allowed to thaw in ice.
2) The volume of components was prepared as follows:

component	Volume reaction (ul) With RNase Inhibitor
10X RT Buffer	2.0
25X dNTP Mix (100mM)	0.8
10X RT Random primers	2.0
Reverse transcriptase	1.0

RNase Inhibitor	1.0
Nuclease- free H_2O	3.2
Total per Reaction	10.0

Table (2): cDNA Reverse Transcription Kit Components

3) 2 X RT master mix was placed on ice and mixed gently.

3.2.6.2.2..Preparation the cDNA Reverse Transcription Reactions:

a) 10 ul of 2 X RT master mix was pipette into tubes.

b) 10 ul of RNA sample was pipette into each tube, pipetting up and down two times for mixing took place.

c) The tubes were sealed.

d) Centrifugation of the tubes to spin down the contents and to eliminate any air bubbles occurred.

e) The tubes were placed on ice until was ready to be loaded on thermal cycler.

3.2.6.2.3. Performing reverse transcription:

1) The thermal cycler was programmed using one of the following:

Condition	Step1	Step 2	Step 3	Step 4
Temperature (^{0}C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Table (3): Conditions and Steps for Thermal Cycler

2) The reaction volume was set to 20 uL.

3) The reactions were loaded into thermal cycler.

4) The reverse transcription run was started.

3.2.6.2.4. Storing cDNA Reverse Transcription Reactions:

The cDNA RT plates or tubes were stored at 2 to 6 ^{0}C for short- term and at -15 to -25 ^{0}C for long-term.

3.2.7. Quantization of mRNA using SYBR Green qPCR: [61]

This was done using the Real-time PCR cycler Rotor Gene Q - QIAGEN for quantization of mRNA using SYBR green kit. The Protocol for SYBR Green qPCR was as followed:

1- Gently vortexing and briefly centrifugation were made to all solutions after thawing.

2- A reaction master mix was prepared by adding the following components (except template DNA) for each 25 ul reaction to a tube at room temperature:

Maxima SYBR Green qPCR Master Mix (2X), no ROX	12.5 ul
Forward Primer	0.3 uM
Reverse Primer	0.3 uM

ROX Solution	10nM/100nM
Template DNA	> 500ng
Water nuclease free	To 25 ul
Total volume	25 ul

Table (4): Components of SYBR Green Kit

3- The master mix was mixed thoroughly and dispersed appropriate volumes into PCR tubes were done.

4- The template DNA (>500 ng/reaction) was added to individual PCR tubes containing the master mix.

5- Gently, the reaction was mixed without creating bubbles (did not vortex). Centrifugation briefly, bubbles would interfere with fluorescence detection.

6- The thermal cycler was programmed according to the recommendations below for four step cycling process; the samples were placed in the cycler and the program was started :

Step	Temperature ^{0}C	Time	Number of cycles
Initial Denaturation	95	10 min	1
Denaturation	94	30 sec	50
Annealing	57	30 sec	
Extension	72	30 sec	

Table (5): Steps, Temperature, Time and Number of Cycles needed for qrt-PCR

3.2.8. Preparation of PLGA Nanoparticles: [62]

3.2.8.1. Materials needed for PLGA nanoparticles preparation:

1- Poly (lactic-co-glycolic acid) (PLGA) [Sigma- Aldrich].
2- Bovine serum albumin fraction.
3- Dichloromethane (DCM).
4- Poly vinyl alcohol (PVA) [Sigma-Aldrich].
5- Ultrapure water from Milli-Q water system.

3.2.8.2. Steps for Preparation of PLGA Nanoparticles:

a) PLGA Polymer was dissolved in organic phase (4ml) then, sonicated with addition of IAP to make primary emulsion.

b) For the formation of secondary emulsion 16 ml of EAP was added drop wise manner into the primary emulsion during sonication of primary emulsion.

c) Then the secondary emulsion was kept in magnetic stirrer for overnight for excess DCM to evaporate.

d) Particles were separated through centrifugation at 15000 rpm for 20 mins.

e) Separated particles were washed twice with ice cold MQ water then particles were lyophilized to obtain dry particles and stored in -20^{o}C for further use.

f) Same methodology was followed in case of PcDNA3.1-VEGF loaded particle formation, but 10ml of EAP was used instead of 16ml and there was no use of sucrose and sodium bicarbonate in IAP.

3.2.8.3. Preparation of PLGA nanoparticles by W/O/W technique:

The preparation of PLGA nanoparticles and Polyplex preparation by W/O/W technique was shown in the following table:

Preparations	PcDNA3.1-VEGF	PLGA	BSA	DCM	MQ – water	PVA
PLGA-NPs alone	Non	0.2 g	Non	4 ml	16 ml	0.16 g
PLGA-NPs - BSA	Non	0.2 g	800ul	4ml	16 ml	0.16 g
PcDNA3.1-VEGF-PLGA-NPs	250 ul	0.125 g	Non	2.5 ml	10 ml	0.19 g

Table (6): Various PLGA nanoparticles Preparations reagents

3.2.8.4. Characterization of PLGA nanoparticles and PLGA measurements:

Morphology, Particle size, Zeta potential were investigated, also XRD of PLGA, FTIR and TGA analysis for PLGA were performed:

3.2.8.4.1. Morphology: [63]

External morphology of nanoparticles was determined using Transmission Electron Microscope (TEM). Samples were prepared by placing one drop on a copper grid, dried under vaccum pressure before being examined using TEM without being stained.

3.2.8.4.2. Particle size analysis: [63]

For analyzing particle size, nano-suspension was diluted with filtered (0.22 um) ultra pure water samples and was analyzed using Zetasizer Nano ZS Size Analyzer (Malvern instrument, UK) which allows sample measurement in the range of 0.020-2000.00 um.

3.2.8.4.3. Zeta potential: [64]

Zeta potential in de-ionized water was determined using Zetasizer Nano ZS size analyzer, Malvern, UK. The samples were prepared by suspending the freeze dried NPs in 5 ml deionized water.

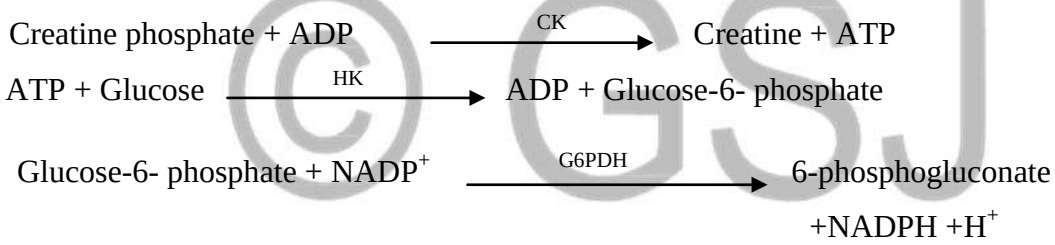
3.2.9. Measuring Cardiac Enzymes of injected and non-injected animals (Cardiac profile):

3.2.9.1. Creatine Kinase (CK): [65]

Creatine Kinase was an enzyme which is contained in heart, brain and skeletal muscles. Thus an increase of circulating level of CK might be associated to myocardial infarction. After a myocardial infarct, CK level begins raising between 4^{th} and 6^{th} hour after first acute symptoms reaching the peak between 18^{th} and 30^{th} hour**.**

3.2.9.1.1. Principle:

CK catalyzed the phosphorylation of ADP, in presence of creatine phosphate to form ATP and creatine. The catalytic concentration was determined from the rate of NADPH formation; measured at 340 nm by means of hexokinase (HK) and glucose- 6- phosphate dehydrogenase (G6PDH) coupled reactions:



3.2.9.1.2. Reagents:

- **Reagent 1 (pH 6.7)(Buffer/ coenzyme)**

Imidazol	125 mmol/L
D- Glucose	25 mmol/L
N- Acetyl -L-Cysteine	25 mmol/L
Magnesium acetate	12.5 mmol/L
NADP	2.5 mmol/L
EDTA	2 mmol/L

- **Reagent 2 (Enzymes)**

ADP	15.2 mmol/L
AMP	25 mmol/L
P1, P5-di (adenosine-5'-) penta-phosphate	103 mmol/L
Glucose- 6- phosphate Dehydrogenase (G6PDH)	9 KU/L

Creatine phosphate	250	mmol/L
Hexokinase	3	KU/L

3.2.9.1.3. Procedure:

1- One milliliters working solution and 50ul serum were pipette into a thermostatized cuvette.

2-Mixing and incubation for 3 minutes took place.

3- Initial absorbance (A) of the sample was read and after 1 minute then after 3 minutes.

4- The difference between absorbances and the average absorbance differences per minute were calculated.

5- ΔA/min X 3333 = U/L CK was performed.

3.2.9.2. Creatine Kinase MB (CK-MB): [66]

CK presented in three different isoenzymatic forms which could be separated by electrophoresis or column chromatography. CK existed in serum in dimeric forms CK-MM, CK-MB, and CK-BB and as macro-enzymes. Measurements of CK-MB was specific test for detection of cardiac muscle damage and therefore used for diagnosis and monitoring of myocardial infarction.

3.2.9.2.1. Principle:

A specific antibody inhibited the M subunits of CK-MM and thus allowed the determination of the B subunit of CK-MB. CK-B catalytic concentration was determined from the rate of NADPH formation, measured at 340 nm by means of hexokinase (HK) and Glucose- 6- phosphate Dehydrogenase (G6PDH) coupled reactions.

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6- phosphate}$$

$$\text{Glucose-6- phosphate} + NADP^{+} \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + NADPH + H^{+}$$

3.2.9.2.2. Reagents:

- **Reagent 1 (pH 6.7)(Buffer/ coenzyme)**

Imidazol	125	mmol/L
D- Glucose	25	mmol/L
N- Acetyl -L-Cysteine	25	mmol/L
Magnesium acetate	12.5	mmol/L
NADP	2.5	mmol/L
EDTA	2	mmol/L

- **Reagent 2 (Enzymes)**

ADP	15.2 mmol/L
AMP	25 mmol/L
P1, P5-di (adenosine-5'-) penta-phosphate	103 mmol/L
Glucose- 6- phosphate Dehydrogenase (G6PDH)	9 KU/L
Creatine phosphate	250 mmol/L
Hexokinase	3 KU/L
Anti- human - CK-M.	

3.2.9.2.3. Procedure:

1- One milliliter working solution and 50ul serum was pipette into a thermostatized cuvette.

2-Mixing and incubation for 3 minutes took place.

3-Initial absorbance (A) of the sample was read and after 1 minute then after 3 minutes.

4-The difference between absorbances and the average absorbance differences per minute were calculated.

5- ΔA/min X 6666 = U/L CK-MB was performed.

3.2.9.3. Lactate dehydrogenase (LDH): [67]

The Lactate dehydrogenase (LDH) enzyme was widely distributed in heart, liver, muscles and kidney. LDH catalyzed the conversion of lactate to pyruvate. The enzyme was a tetrameric protein and gave rise to five isoenzymes. LDH was significantly increased during myocardial infarction.

3.2.9.3.1. Principle:

LDH catalyzed the reaction between pyruvate and NADH to produce NAD^+ and L-Lactate:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + NAD^+$$

The initial rate of NADH oxidation was directly proportional to the catalytic LDH activity and was determined by measuring the decrease in absorbance at 340 nm.

3.2.9.3.2. Reagents:

Reagent 1 (R1 Buffer)	
Tris buffer (pH 7.5)	50 mmol/L
Pyruvate	3 mmol/L
Sodium Azide	8 mmol/L
Reagent 2 (R2 coenzyme)	
NADH	> 0.18 mmol/L
Sodium azide	8 mmol/L

3.2.9.3.3. Procedure:

1- one milliliter of working solution was added to 20 ul specimen.

2- Mixing occurred and measuring of absorbance after 30 seconds took place.

3- Reading after 1, 2, and 3 minutes was applied and mean absorbance change per minute was performed.

4- LDH activity was calculated using the following formula U/L = 8095 x ΔA at 340 nm/min.

3.2.10. Investigation of the myocardium:

The Histopathological Study was as follows:

Paraffin sections were made from Parts of cardiac tissues of rats in each group which were processed and stained by conventional haematoxylin and eosin (H&E) stains for studying the histological changes of the cardiac tissues as follows:

1. The cardiac tissue was fixed at 10% ne formaldehyde.
2. Then dehydration in ascending grades of alcohol (70%- 100) was done.
3. Clearing in xylene took place.
4. Impregnation in molten paraffin at 60 ^{O}C degrees for 1-2 hrs to produce paraffin block was made.
5. The blocks were cut into 5 µm thick sections with a microtome. These sections were floated in water bath, picked up in clean glass slides and then left in 40 ^{O}C degrees for dry and fixed sections on slides.
6. The paraffin sections were depraffinized with xylene then rehydrated in descending grades of alcohol (100%-70%).
7. The slides were rinsed in haematoxylin stain for one minute and washed in tap water to differentiate the violet color and remove excess of color, then rinsed in eosin stain for 5 minutes.
8. Then slides were dehydrated in the ascending series of alcohol and cleaned with xylene.

The slides were mounted by DPX and Covered by covering slides, thus the slides were ready to be examined by light microscopy and Photographed using Olympus light microscope – equipped with Spot digital camera and computer program software in Histopathological Unit of Medical Technology Center - .Medical Research Institute - Alexandria University.

3.2.11. Analyzing the QRT-PCR Data using Comparative CT Method (ΔΔCT):

The data obtained from an experiment where put in a table, where the expression levels of a target gene VEGF and an endogenous control B-actin are evaluated. The levels of these amplicons in a series of drug-treated samples are compared to an untreated calibrator sample.

The number of experimental replicates run in a study directly affected the downstream data analysis (i.e. were the observed fold-differences in nucleic acid statistically significant. Careful consideration might be exercised when determining the number of experimental replicates that would be tested in a relative quantitation study. Mean CT values and standard deviations were used in the ΔΔCT calculations. CT mean was calculated and standard deviations were calculated for each mean

CT value. Accordingly Fold change expression of VEGF after treatment, was calculated by ΔΔCT method as follows:

3.2.11.1. Calculations of the ΔCT value:

The ΔCT value was calculated by subtraction of the average B-actin CT value from the average VEGF CT value of the treated and untreated samples.

ΔCT = CT target gene (VEGF) – CT reference gene (B-actin)

3.2.11.2. Calculations of the ΔΔCT value:

The ΔΔCT was calculated by:

ΔΔCT = ΔCT test sample – ΔCT calibrator sample i.e. by subtracting the ΔCT of the untreated from the ΔCT of Drug Treatment.

At this point we got the true fold change; we took the log base 2 of this value to even out the scales of up regulated and down regulated genes. Otherwise up regulated has a scale of 1-Infinity while down regulated has a scale of 0-1.

Based on your amplification plots, the computer would determine the best threshold to set whereby the most amplification plots were in a linear growth phase. Once the threshold was set, the cycle at which each amplification curve crossed that threshold was determined and assigned as the CT for that sample. With this data, groups were obtained and proceeded to calculate the change in expression values for each gene in cardiac myocytes.

The CT data was used to determine the amount of each gene/mRNA presented relative to each sample. ΔΔCT and the relative amount of VEGF mRNA in terms of fold change. ΔCT was calculated by Subtracting the CT for VEGF for the sample from the CT for the endogenous control, and evaluating the fold expression: $2^{-\Delta\Delta CT}$ took place. Data were graphed in a variety of ways, once expression has been determined, for easier visualization.

3.2.12. Statistical analysis of the data:

Data were fed to the computer using IBM SPSS software package version 20.0.

Qualitative data were described using number and percent. Comparison between different groups regarding categorical variables was tested using Chi-square test.

Quantitative data were described using mean and standard deviation for normally distributed data while abnormally distributed data was expressed using median, minimum and maximum.

For normally distributed data, comparison between two independent population were done using independent t-test while more than two population were analyzed F-test (ANOVA) to be used.

Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

Mean value $(\overline{X}) = \frac{X}{n}$.

Where X = the sum of all observations.

n = the number of observations.

The standard deviation S.D. = $\sqrt{\frac{\sum (X - \overline{X})^2}{n-1}}$

Where

$\Sigma (Xi - X)^2$ = the sum of squares of differences of observations from the mean.

One way analysis of variance (ANOVA) was performed for comparison between more than two groups

Variance ratio F was computed by the formula.

$$F_{(r-1),(n-1)} = \frac{Mean\ square\ between\ classes}{Mean\ \ square\ within\ classes}$$

Where r = number of groups

n = total sample size

4-Results

4.1. Agarose gel electrophoresis for PcDNA3.1-VEGF:

Plasmid DNA was isolated from *E.coli*, using Mini preparation (Mini prep.) method. The isolated plasmid DNA was run on 1% agarose gel electrophoresis to check the integrity of the plasmid. The isolated plasmid was super coiled in conformation. The plasmid had Ampicillin resistance gene to serve as selection marker. Picture of gel with plasmid DNA and marker DNA are shown in the following figure (4.1). Then plasmid DNA was purified using the plasmid purification kit. The concentration and purity of plasmid DNA were determined by Nanodrop device (Jenway). Usually three bands of PCDNA3.1-VEGF appeared in the Agarose gel (marker is 1K), the highest band is for circular plasmid, the middle is for linear plasmid and the lowest is for super coiled plasmid. Each form of plasmid had its migration rate corresponding to different position in agarose gel and had a certain molecular weight.

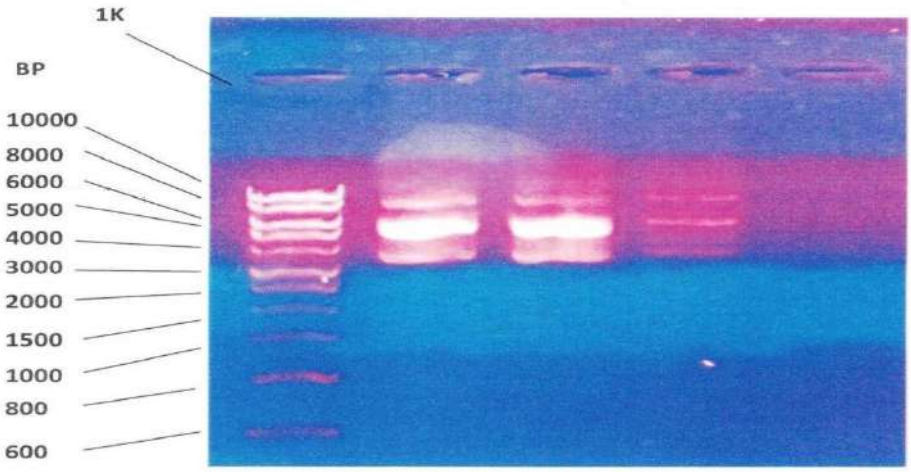


Figure 4: Agarose gel electrophoresis of PcDNA 3.1 – VEGF.

Concentration of PcDNA3.1-VEGF ug/ml	Purity (260/280)	Purity (260/230)
372.62	1.776	0.916

Table (7): Concentration and purity of PcDNA3.1 -VEGF

4.2. Characterization of PLGA- NPs:

4.2.1. Image and Particle size of PLGA- nanoparticles using TEM:

4.2.1.1. TEM for PLGA- NPS alone:

This was done using Jsm 1400 -+ Jeol Japan Transmission Electron Microscope as shown below:

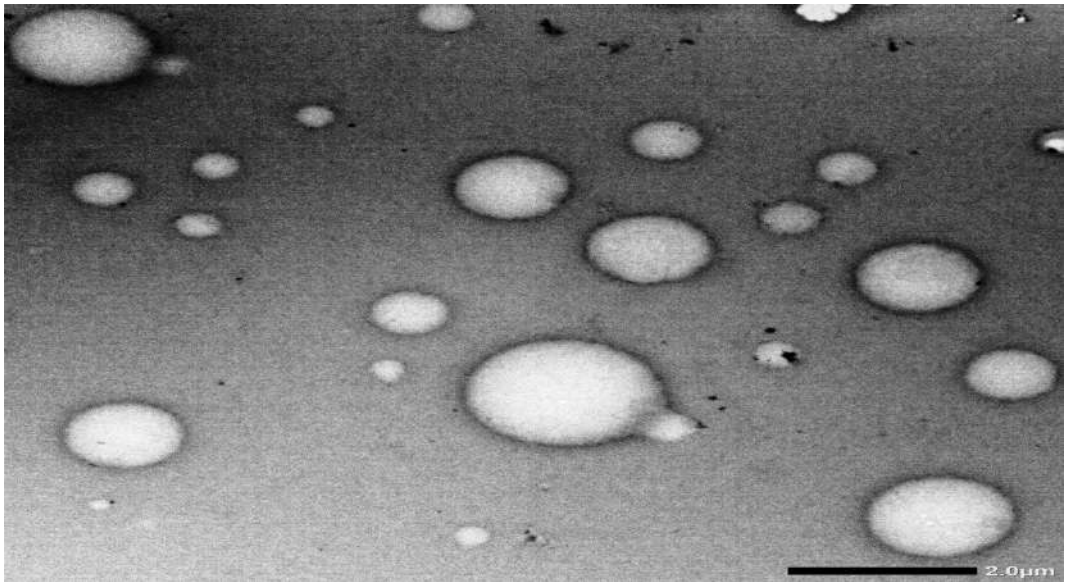


Figure 5: TEM image for PLGA- NPs alone.

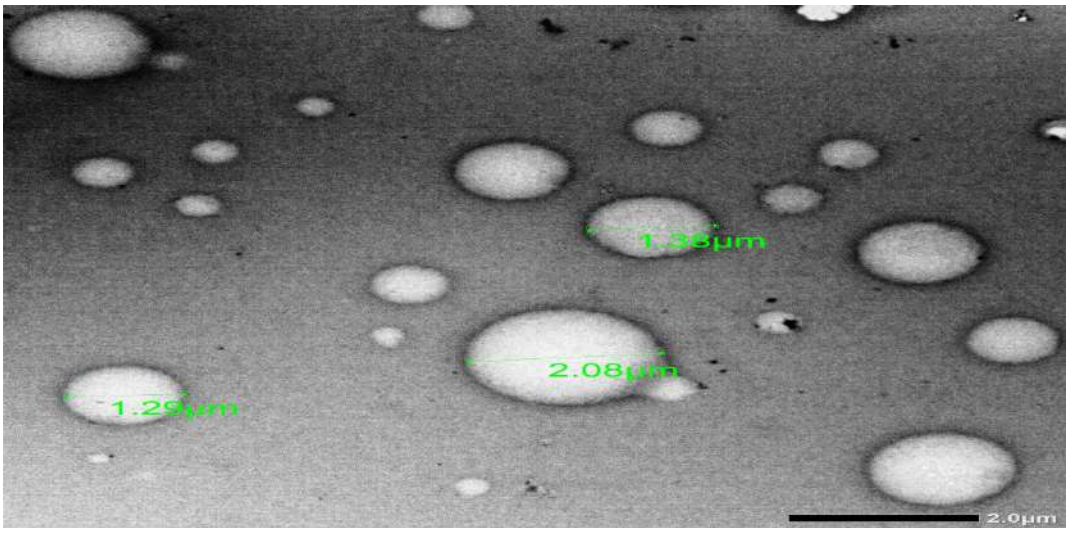


Figure 6: The Particle size for PLGA-NPs alone.
(Mean particle size is 1.58 um), which was larger than with carrier protein (BSA).

4.2.1.2. TEM for PLGA - NPs- BSA:

This was done using Jsm 1400 -+ Jeol Japan Transmission Electron Microscope as shown below:

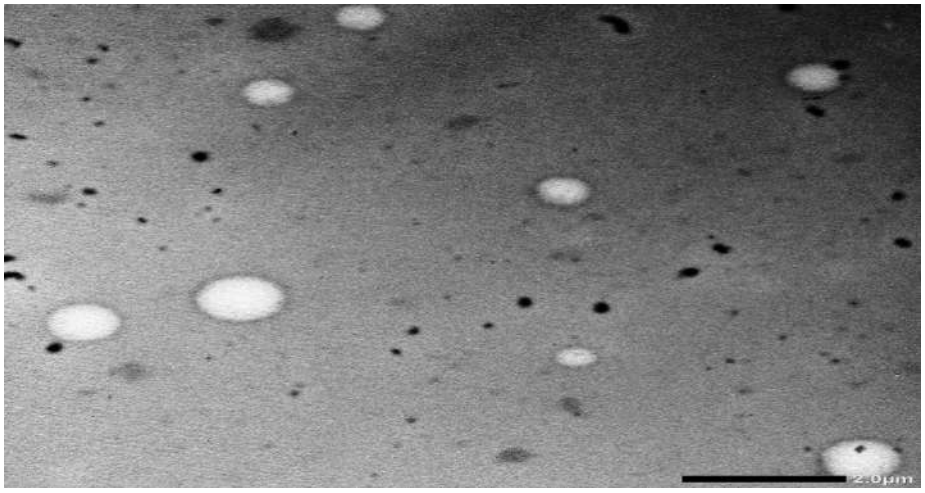


Figure 7: TEM image for PLGA-NPs – BSA.

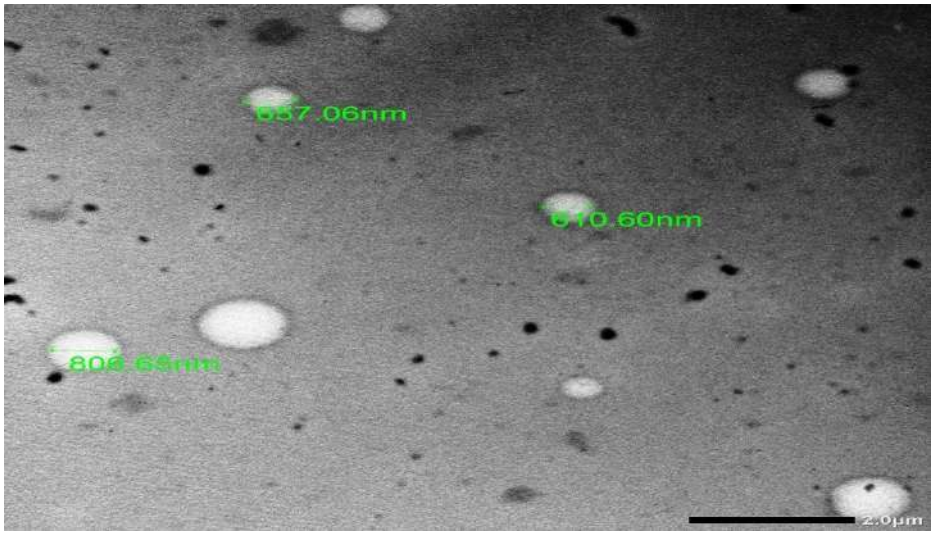


Figure 8: Particle size of PLGA-NPs-BSA.
(The mean particle size is: 691.4 nm), which was much smaller than that the nano-particle of PLGA alone.

4.2.1.3. <u>TEM for PcDNA3.1 carrying VEGF and PLGA-NPs complex:</u>

This was done using Jsm 1400 -+ Jeol Japan Transmission Electron Microscope as shown below:

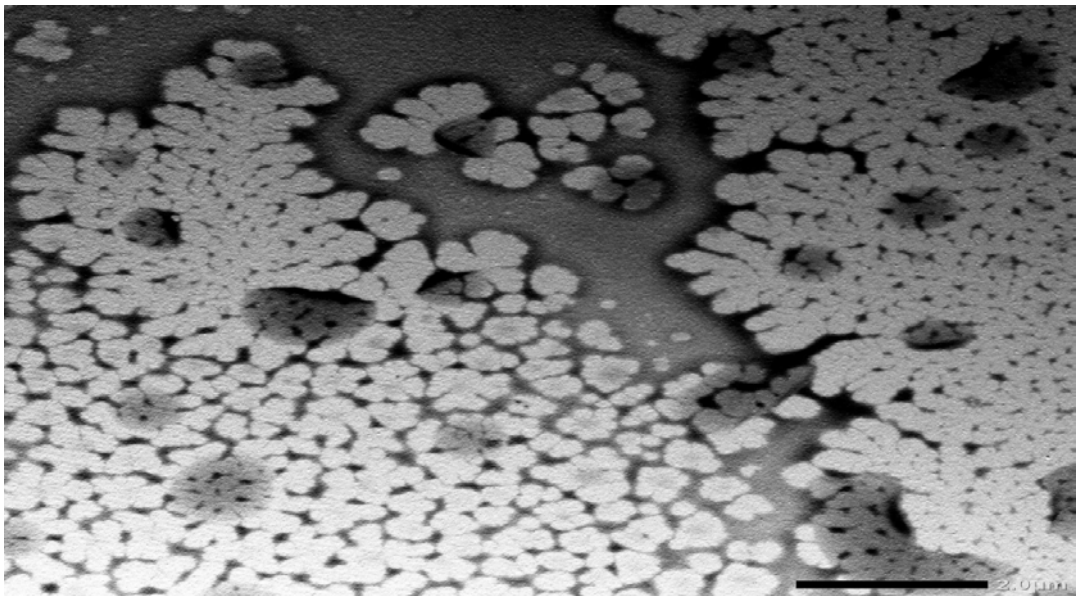


Figure 9: TEM image of the gene transfer complex (polyplex), which was formed by the interaction between PcDNA3.1 carrying VEGF and PLGA-NPs.

4.2.2. <u>Zeta-sizer and Zeta potential</u>: (3 samples were measured by each).

PLGA nanoparticles were prepared by double solvent evaporation method. Then BSA (2.5%) was used as a model protein and PcDNA 3.1 –VEGF was used as a DNA carrier for evaluation of the properties of the nanoparticles. The particle size was determined by Zeta sizer Nano ZS Size Analyzer Malvern device. Particles which were prepared by sonication for both primary and secondary emulsion were in nanometer range.

For PLGA polymer NPs, the particle size was measured. Respective PDI (polydispersity index) values of the formulations were evaluated. Polydispersity index (PDI), a term in polymer chemistry referring to the molecular weight distribution of polymers. PDI is the mass average degree of molecular weight to the number average degree of molecular weight. Zeta potential is the scientific term used for electro-kinetic potential. The significance of zeta potential indicates that its value is related to colloidal dispersions stability.

The zeta potential measures degree of repulsion between adjacent, charged particles in dispersion. For small enough molecules and particles, a high zeta potential indicates stability, and solution or dispersion will resist aggregation process. At low potential, attraction exceeds repulsion and breaking and flocculation of dispersion takes place. So, colloids having high zeta potential (negative or positive) are electrically stable, while colloids with low zeta potentials have tendency for coagulation or flocculation.

Sample 1 was the normal sample in which there was no IAP (BSA/PcDNA3.1-VEGF), the particle size was measured. When BSA was added to the particles (sample 2), the size differs and was seen in the following table also The detail parameters like particle size, zeta potential, PDI and loading efficiency were shown as given in table:

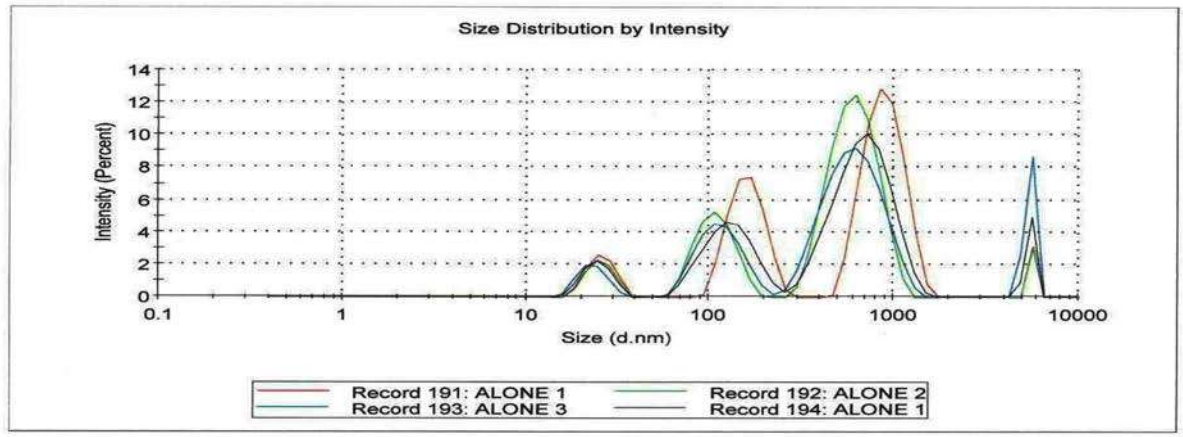
Sample	Peaks	size	% intensity	St. Dev.	Z-average	Intercept	PdI
PLGA-NPs alone	Peak 1	697.6	61.4	233.1	500.8	0.970	0.890
	Peak 2	131.0	25.4	39.39			
	Peak 3	24.63	7.4	4.296			
PLGA-NPs-BSA	Peak 1	863.1	58.9	149.8	2494	0.969	1.000
	Peak 2	414.8	29.1	39.70			
	Peak 3	33.86	7.6	3.861			
PcDNA3.1-VEGF – PLGA-NPs	Peak 1	694.9	89.1	126.0	1422	0.877	0.946
	Peak 2	78.01	10.9	17.72			
	Peak 3	0.000	0.0	0.000			

Table (8): Size and % intensity values of different samples using Malvern device

Sample	Peaks	Mean	Area (%)	St. Dev.	Zeta Potential	Zeta Deviation(mv)	Conductivity (mS/cm)
PLGA-NPs alone	Peak 1	-3.25	53.1	3.96	-11.9	10.6	0.0354
	Peak 2	-21.7	46.1	5.84			
	Peak 3	0.00	0.0	0.00			
PLGA-NPs-BSA	Peak 1	2.85	100	4.42	2.85	4.42	0.0645
	Peak 2	0.00	0.0	0.00			
	Peak 3	0.00	0.0	0.00			
PcDNA3.1-VEGF – PLGA-NPs	Peak 1	-14.3	100	6.86	-14.3	6.86	0.0243
	Peak 2	0.00	0.0	0.00			
	Peak 3	0.00	0.0	0.00			

Table (9): Zeta potential and Zeta deviation values of different samples using Malvern device

The following curves represented Zeta-sizer measurements and Zeta potential which was as follows:



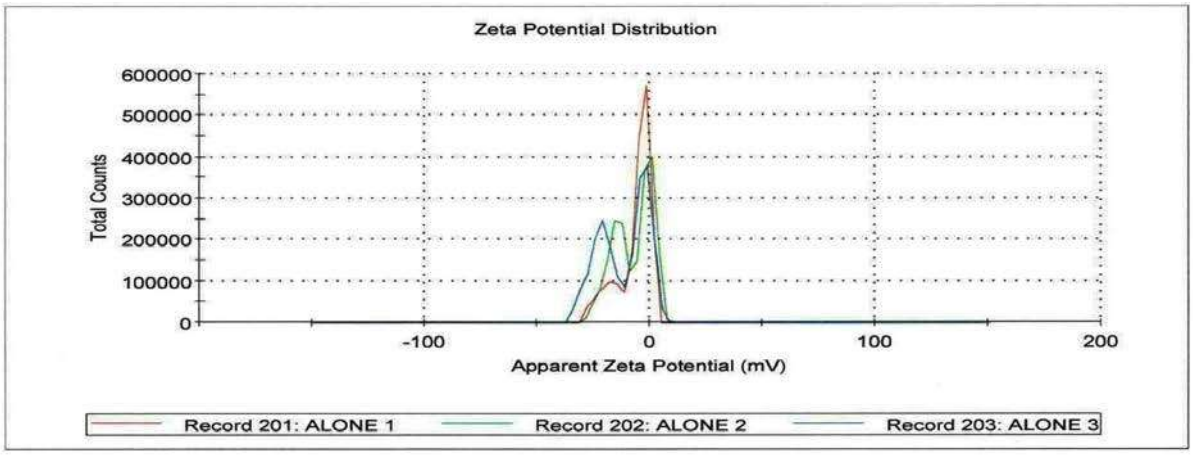


Figure 10: Size Distribution and Zeta potential For PLGA- NPs alone sample.

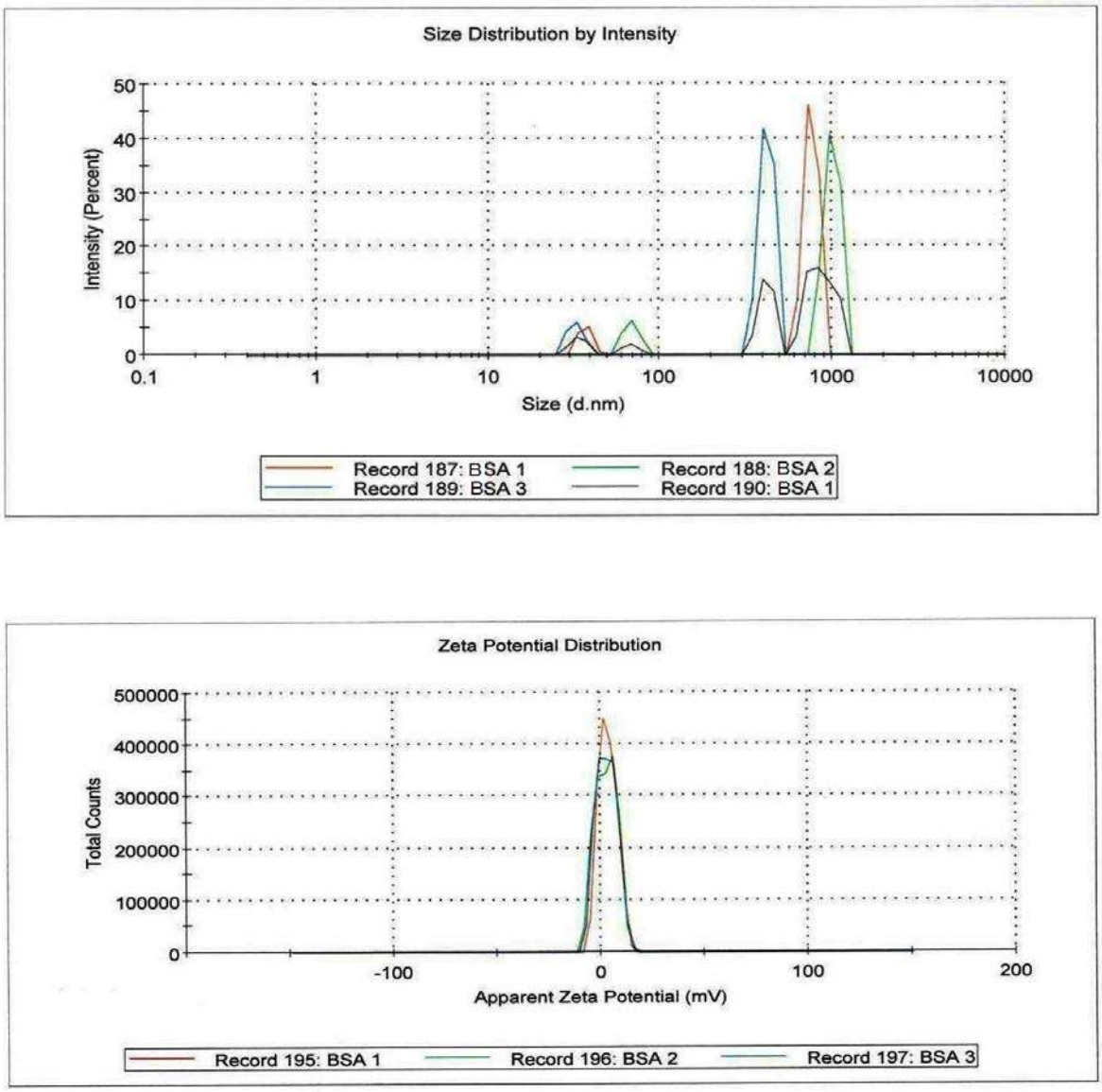


Figure 11: Size Distribution and Zeta potential For PLGA- NPs – BSA sample.

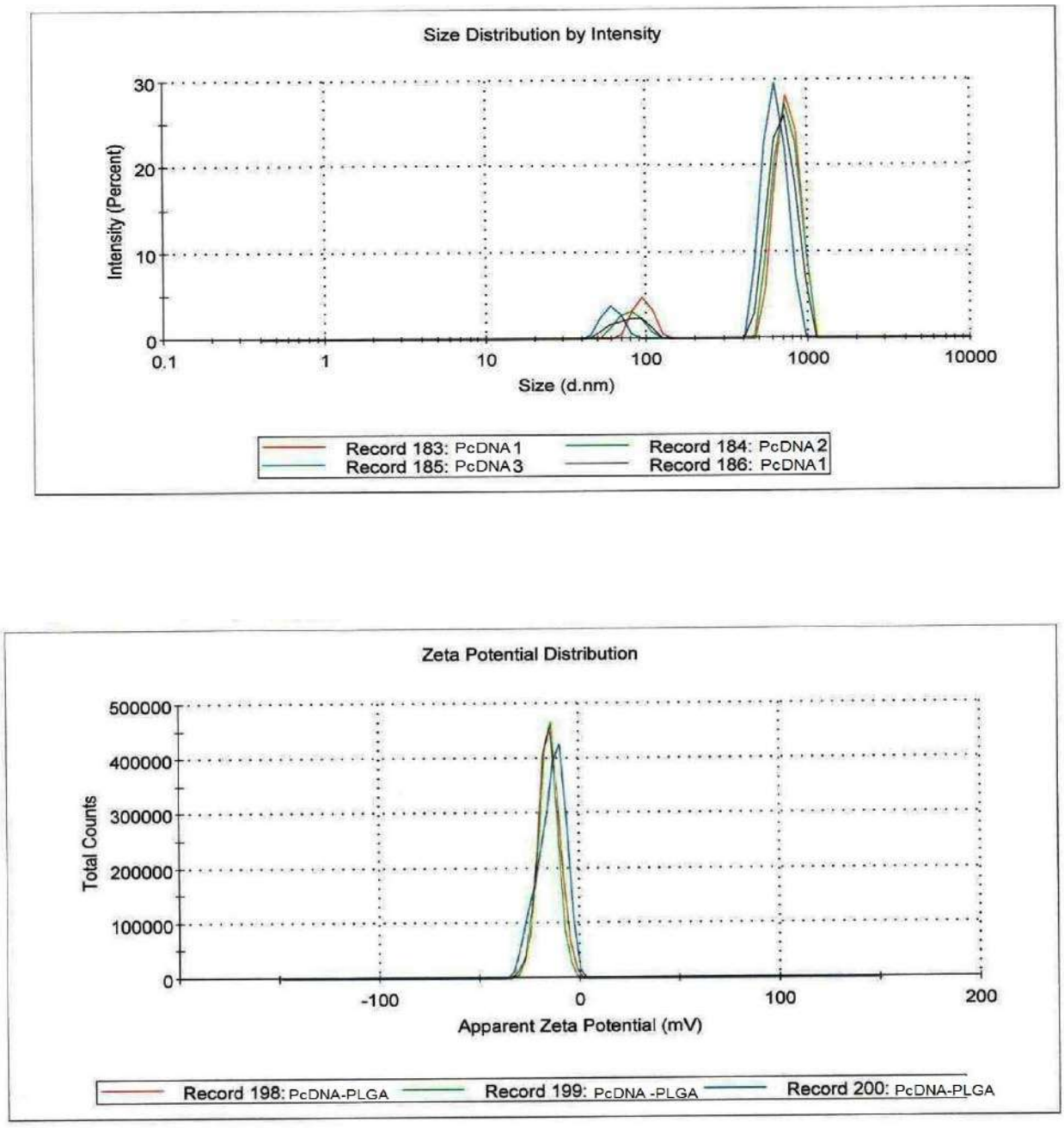


Figure 12: Size Distribution and Zeta potential for PcDNA3.1-VEGF- PLGA- NPS.

The following table showed comparison between the studied groups of rats regarding their weight. There was no statistical significant difference between the three studied groups' rates regarding their weight ($p > 0.5$):

	Normal Healthy	Injection with Isoprenaline HCl to induce	PcDNA-VEGF alone (T1)	PLGA- NPs – BSA (T2)	PcDNA3.1-VEGF-PLGA-NPs

		MI (D)			complex (T3)
Range	350.0-391.0	350.0-391.0	369.0-391.0	338.0-380.0	349.0-388.0
Mean	371.25	371.25	381.00	361.00	376.25
S.D.	19.02	19.02	9.09	18.49	18.34
F	0.745				
p	0.576				

Table (10): Comparison between the studied groups rats regarding their weight.

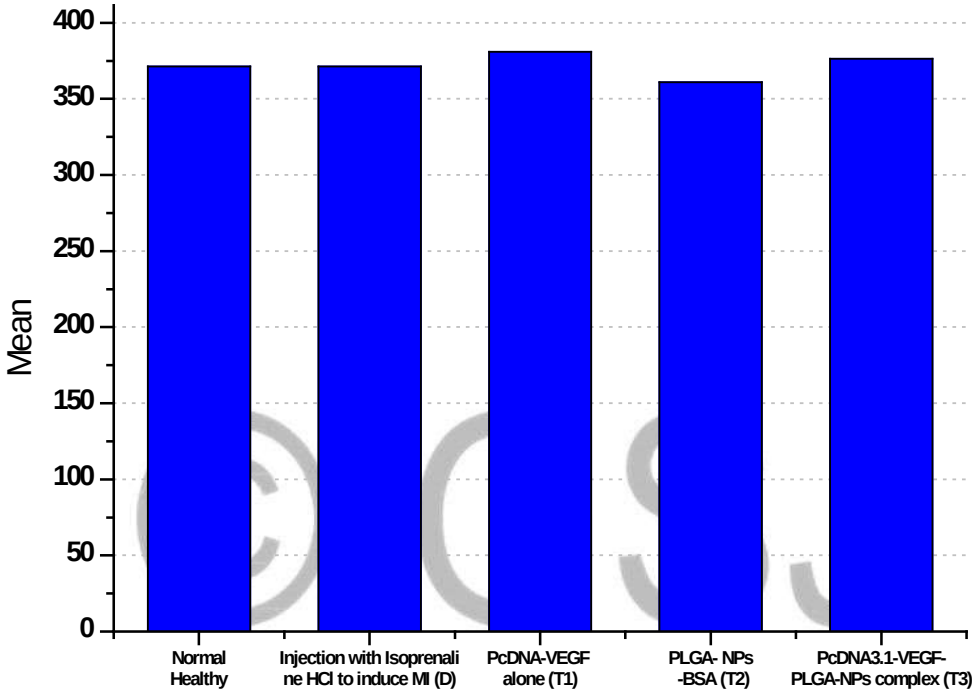


Figure 13: Comparison between the studied groups rats regarding their weight.

Injected Material (One Rat/group)	Duration	Cardiac Profile (Parameters)		
		CK (U/L)	CK-MB (U/L)	LDH (U/L)
Non-injected normal healthy animals (Negative control)	Immediately	120±10.82	21±2.1	240±22.1
Injected with Isoprenaline HCl (Positive control)	After one day	215±18.9	35±2.89	490±42.8
Injected with PcDNA3.1- VEGF	After 3d	200±17.6	28±2.67	380±29.8
	After 5d	180±15.92	25.5±1.98	365±31.2

alone (T1)	**After 7d**	**150±15.4**	**23±2.14**	**320±28.4**
Injected with PLGA-NPs-BSA (T2)	**After 3d**	**213.5±20.3**	**30±3.10**	**485±41.5**
	After 5d	**212±18.6**	**29±2.78**	**475±42.3**
	After 7d	**209±19.8**	**27±2.66**	**466±41.6**
Injected with PcDNA3.1-VEGF-PLGA-NPs complex (T3)	**After 3d**	**180±17.6**	**26±2.71**	**330±31.7**
	After 5d	**135±12.3**	**24.2±2.36**	**300±24.5**
	After 7d	**125±11.5**	**22±1.98**	**270±25.8**

Table (11): Cardiac Parameters (profile) of Injected and non- injected animals within treatment course (Reference Ranges cardiac profile parameters were for CK: 22-198 U/L, CK-MB: Up to 25 U/L and LDH 230-460 U/L).

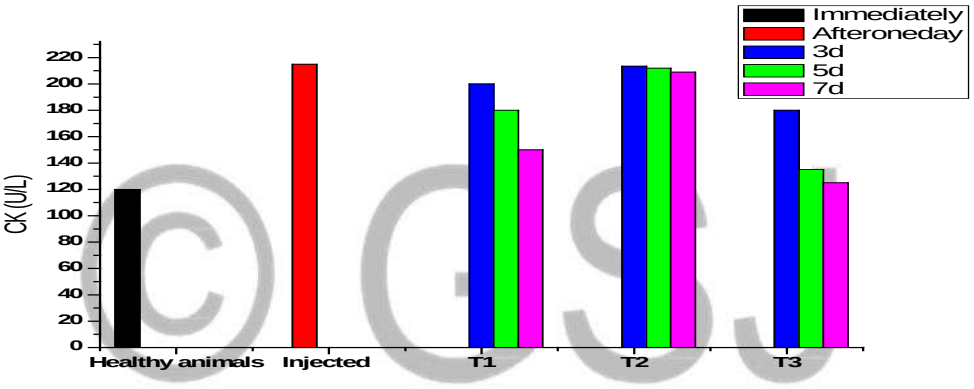


Figure 14: Graphical representation of CK (U/L) levels for all groups.

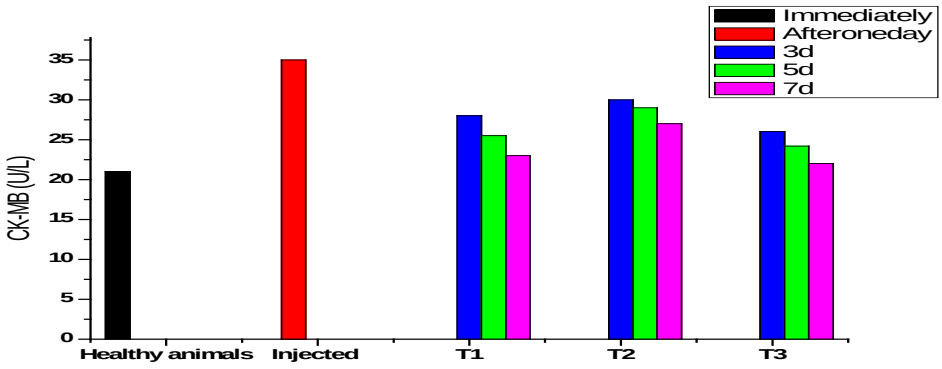


Figure 15: Graphical representation of CK-MB (U/L) levels for all groups.

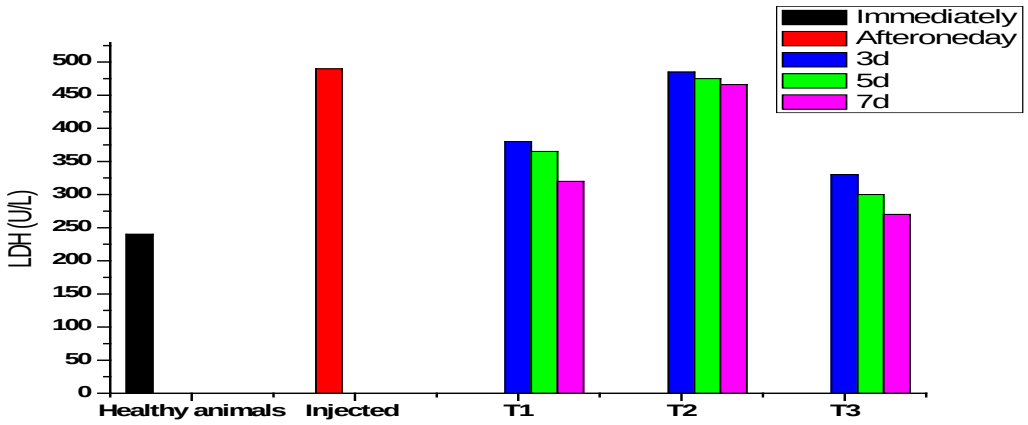


Figure 16: Graphical representation of LDH (U/L) levels for all groups.

The following table showed comparison between different studied groups regarding the RNA concentration. After 3 days, PLGA-NPs – BSA was higher RNA concentration than PcDNA3.1-VEGF and PcDNA3.1-VEGF-PLGA-NPs Complex treatment. After 5 days, PcDNA3.1-VEGF-PLGA-NPs Complex was higher than PLGA-NPs – BSA and PcDNA3.1-VEGF treatment. After 7 days, PLGA-NPs – BSA was higher RNA concentration than PcDNA3.1-VEGF and PcDNA3.1-VEGF-PLGA-NPs Complex treatment. There was statistical significant difference between studied groups regarding the RNA concentration ($P < 0.05$).

	Normal healthy	Myocardial Infarcted animals	Treated groups			p-value
			PcDNA3.1-VEGF	PLGA-NPs – BSA	PcDNA3.1-VEGF-PLGA-NPs Complex	
Min. Max. Mean S.D.	100.0 200.0 156.0 47.30	122.0 290.0 209.75 70.03				
After 3days Min. Max. Mean S.D.			204.0 910.0 550.0 347.25	260.0 1052.0 659.25 414.44	204.0 860.0 498.0 273.86	8.98 0.013*
After 5 days Min. Max. Mean S.D.			119.8 310.0 203.45 95.90	108.0 369.0 213.50 117.56	170.0 970.0 643.75 370.62	12.54 0.009*
After 7 days Min. Max. Mean S.D.			104.0 160.0 124.0 24.78	142.0 960.0 415.75 368.76	135.0 878.0 410.0 324.84	7.98 0.014*

ANOVA P2 value			**19.8 0.0021***	**25.65 0.001***	**23.65 0.0019***	

Table (12): Comparison between different studied groups regarding the RNA concentration (ug/ml)

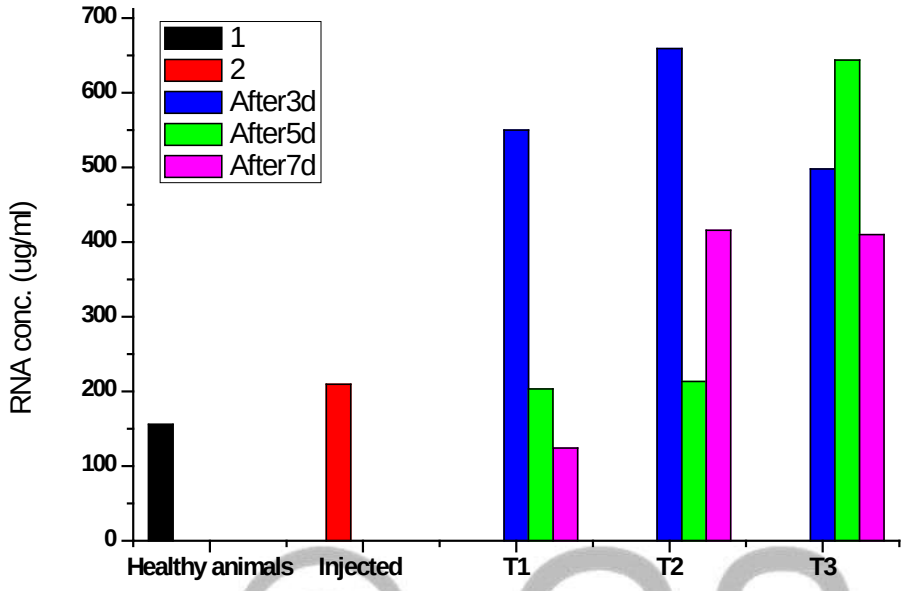


Figure 17: Comparison between different studied groups regarding the RNA concentration (ug/ml).

The following table showed the Comparison between different studied groups regarding the Purity 260/280. There was no statistical significant difference between studied groups regarding the Purity 260/280 at three period (after 3, 5 and 7 days) (P >0.05).

	Normal healthy	Myocardial Infarcted animals	Treated groups			ANOVA p-value
			PcDNA3.1-VEGF	PLGA-NPs – BSA	PcDNA3.1-VEGF-PLGA-NPs Complex	
Min. Max. Mean S.D.	2.00 3.00 2.25 0.50	2.00 4.90 3.43 1.65				
After 3days Min. Max. Mean S.D.			2.20 2.30 2.25 0.06	2.01 2.20 2.11 0.10	2.02 2.50 2.16 0.23	1.01 0.32
After 5 days Min. Max. Mean S.D.			2.10 5.10 3.03 1.40	2.00 2.20 2.11 0.11	2.00 2.70 2.23 0.32	1.25 0.165
After 7 days Min. Max. Mean S.D.			2.10 2.40 2.28 0.13	2.00 3.10 2.30 0.54	2.00 2.40 2.15 0.17	0.98 0.365
ANOVA P2 value			1.89 0.14	0.65 0.58	0.45 0.61	

Table (13): Comparison between different studied groups regarding the Purity 260/280 nm

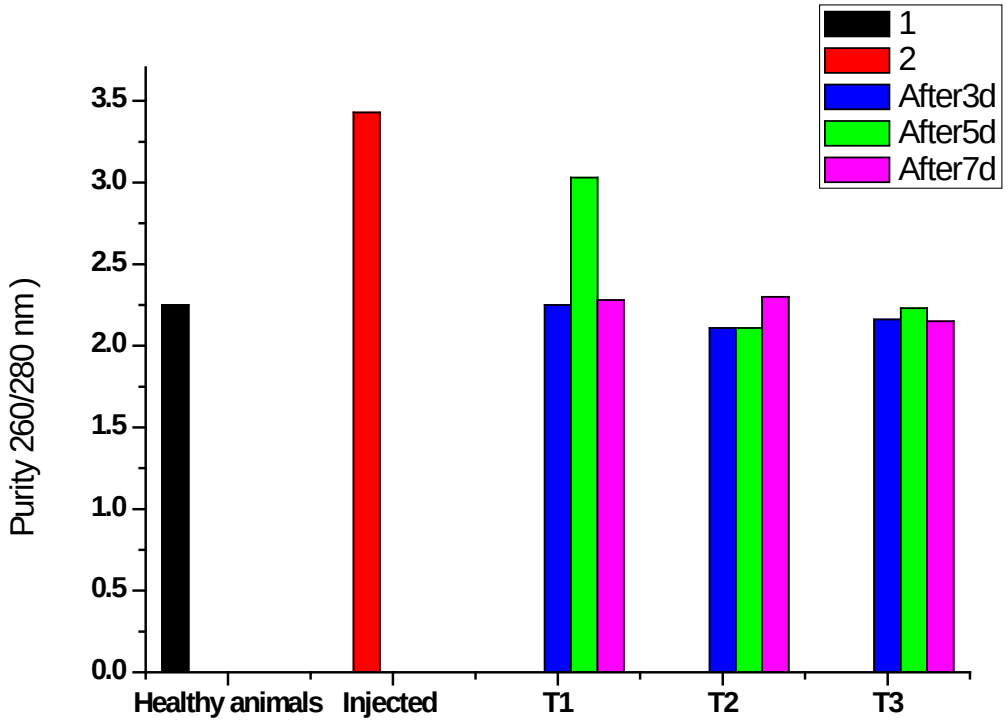


Figure 18: Comparison between different studied groups regarding the Purity 260/280 nm.

The following table showed comparison between different studied groups regarding the Purity 260/230. After 3 days, PLGA-NPs – BSA was higher RNA concentration than PcDNA3.1-VEGF and PcDNA3.1-VEGF-PLGA-NPs Complex treatment. After 5 days, PcDNA3.1-VEGF was higher than PcDNA3.1-VEGF-PLGA-NPs Complex and PLGA-NPs – BSA treatment. After 7 days, PcDNA3.1-VEGF was higher than PcDNA3.1-VEGF-PLGA-NPs Complex and PLGA-NPs – BSA treatment. There was statistical significant difference between studied groups regarding the Purity 260/230 at period (after 3 days and after 7 days) ($P < 0.05$), while there was no statistical significant difference after 5 days ($P > 0.05$).

	Normal healthy	Myocardial Infarcted animals	Treated groups			ANOVA p-value
			PcDNA3.1-VEGF	PLGA-NPs – BSA	PcDNA3.1-VEGF-PLGA-NPs Complex	
Min.	**0.40**	**0.10**				
Max.	**1.70**	**0.20**				
Mean	**0.85**	**0.13**				
S.D.	**0.61**	**0.05**				
After 3days						
Min.			**0.80**	**0.40**	**0.40**	
Max.			**2.50**	**2.20**	**0.80**	**6.22**
Mean			**1.50**	**1.58**	**0.50**	**0.023***
S.D.			**0.80**	**0.81**	**0.20**	
After 5 days						
Min.			**0.20**	**0.80**	**0.10**	**3.25**
Max.			**2.40**	**1.30**	**1.80**	**0.102**
Mean			**1.53**	**0.93**	**1.08**	
S.D.			**0.94**	**0.25**	**0.71**	
After 7 days						
Min.			**1.20**	**0.30**	**0.20**	**5.21**
Max.			**3.30**	**0.80**	**2.00**	**0.036***
Mean			**2.50**	**0.68**	**0.73**	
S.D.			**0.98**	**0.25**	**0.85**	
ANOVA2			**2.65**	**1.98**	**4.65**	
P2 value			**0.069**	**0.107**	**0.036***	

Table (14): Comparison between different studied groups regarding the Purity 260/230 nm

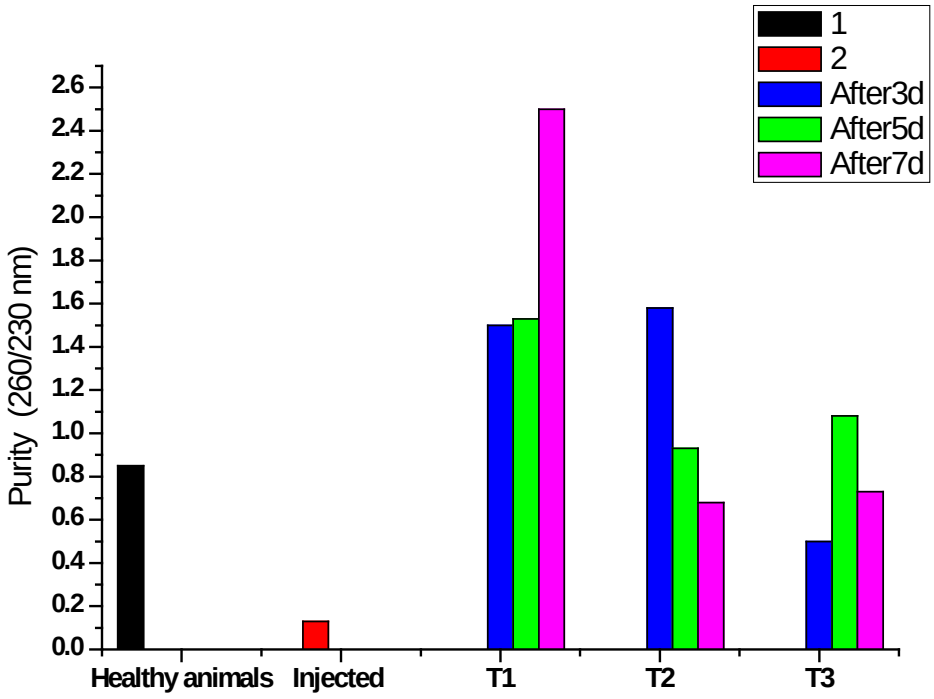


Figure 19: Comparison between different studied groups regarding the Purity 260/230 nm.

The following table showed that PcDNA3.1-VEGF-PLGA-NPs Complex treatment was higher than PcDNA3.1-VEGF and PLGA-NPs – BSA treatments at all periods (after 3, 5 and 7 days):

	Normal healthy	Myocardial Infarcted animals	Treated groups		
			PcDNA3.1-VEGF	PLGA-NPs – BSA	PcDNA3.1-VEGF-PLGA-NPs Complex
Min.	**0.76**	**0.48**			
Max.	**1.16**	**0.74**			
Mean	**1.01**	**0.60**			
S.D.	**0.18**	**0.12**			
After 3days					
Min.			**1.79**	**0.59**	**2.04**
Max.			**2.83**	**1.30**	**3.68**
Mean			**2.24**	**0.93**	**2.74**
S.D.			**0.52**	**0.32**	**0.83**
After 5 days					
Min.			**7.57**	**0.48**	**31.34**
Max.			**11.88**	**1.71**	**38.32**
Mean			**9.02**	**1.00**	**33.53**
S.D.			**1.94**	**0.53**	**3.24**
After 7 days					
Min.			**31.34**	**0.91**	**53.45**
Max.			**38.59**	**2.75**	**145.01**
Mean			**34.77**	**1.56**	**91.65**
S.D.			**3.27**	**0.81**	**41.46**

Table (15): Statistical descriptive analysis of fold expression in all studied groups

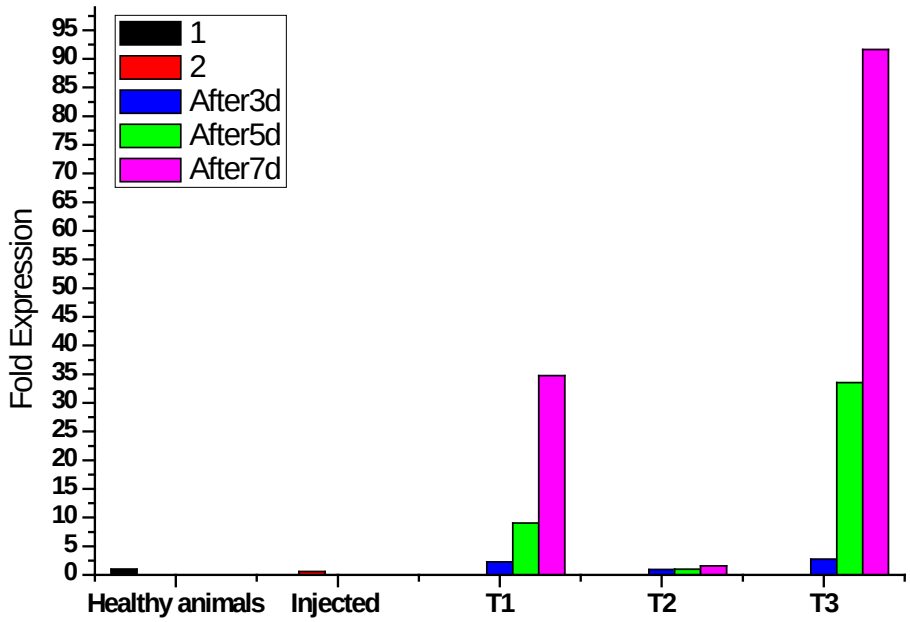


Figure 20: Comparison between different studied groups regarding the fold expression.

The following table shows comparison between fold expression in different treatments after 3 days in relation to normal healthy and myocardial infarcted animals. There was statistical significant difference between normal healthy and other groups (P1 < 0.05) except PLGA-NPs – BSA treatment, between myocardial infarcted and other groups (P2 < 0.05), between PcDNA3.1-VEGF and treatment PLGA-NPs– BSA (P3<0.05) and between PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex (P4< 0.05).

	Normal healthy	Myocardial Infarcted animals	Treated groups (after 3 days)		
			PcDNA3.1-VEGF	PLGA-NPs – BSA	PcDNA3.1-VEGF-PLGA-NPs Complex
Min. Max. Mean S.D.	0.76 1.16 1.01 0.18	0.48 0.74 0.60 0.12	1.79 2.83 2.24 0.52	0.59 1.30 0.93 0.32	2.04 3.68 2.74 0.83
ANOVA P	15.25 0.0031*				
P1		0.014*	0.013*	0.211	0.0062*
P2			0.001*	0.028*	0.017*
P3				0.005*	0.216
P4					0.007*

Table (16): Comparison between fold expression in different treatments after 3 days in relation to normal healthy and myocardial infarcted animals

Where, P1 comparison between normal healthy and other groups
P2 comparison between myocardial infarcted and other groups
P3 comparison between PcDNA3.1-VEGF and treatment PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex
P4 comparison between PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex

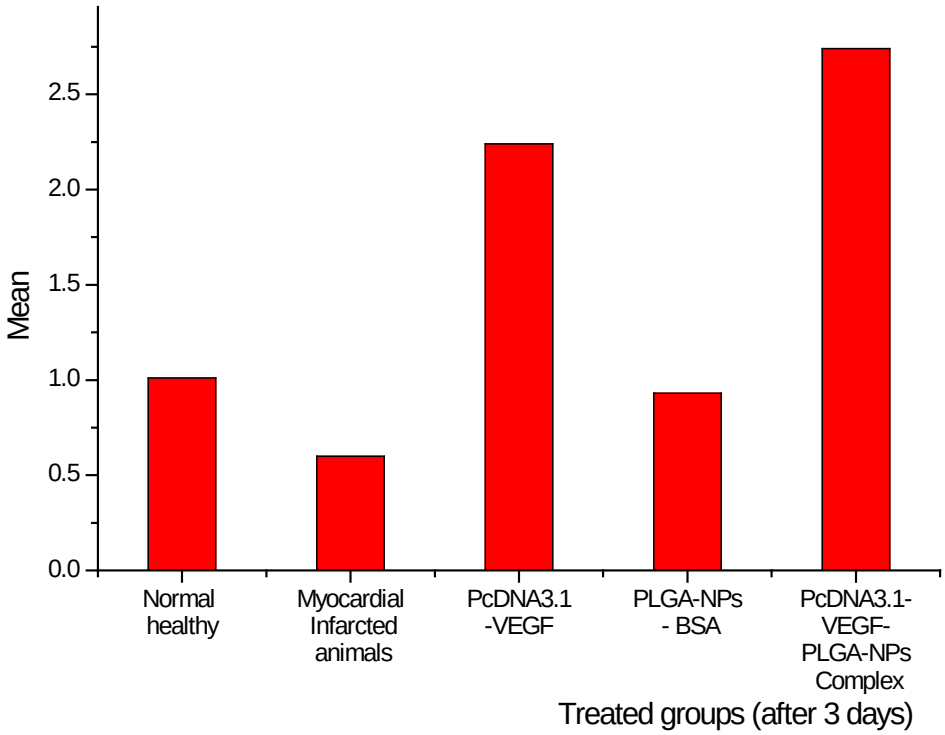


Figure 21: Comparison between fold expression in different treatments after 3 days in relation to normal healthy and myocardial infarcted animals.

The following table showed comparison between fold expression in different treatments after 5 days in relation to normal healthy and myocardial infarcted animals. There was statistical significant difference between normal healthy and other groups (P1 < 0.05) except PLGA-NPs – BSA treatment, between myocardial infarcted and other groups (P2 < 0.05), between PcDNA3.1-VEGF with treatment PLGA-NPs– BSA and PcDNA3.1-VEGF-PLGA-NPs Complex (P3<0.05) and between PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex (P4< 0.05).

	Normal healthy	Myocardial Infarcted animals	Treated groups (after 5 days)		
			PcDNA3.1-VEGF	PLGA-NPs – BSA	PcDNA3.1-VEGF-PLGA-NPs Complex
Min. Max. Mean S.D.	0.76 1.16 1.01 0.18	0.48 0.74 0.60 0.12	7.57 11.88 9.02 1.94	0.48 1.71 1.00 0.53	31.34 38.32 33.53 3.24
ANOVA P	28.5 0.001*				
P1		0.014*	0.001*	0.685	0.001*
P2			0.001*	0.026*	0.001*
P3				0.001*	0.001*
P4					0.001*

Table (17): Comparison between fold expression in different treatments after 5 days in relation to normal healthy and myocardial infarcted animals

P1 comparison between normal healthy and other groups
P2 comparison between myocardial infarcted
P3 comparison between PcDNA3.1-VEGF and treatment PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex
P4 comparison between PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex

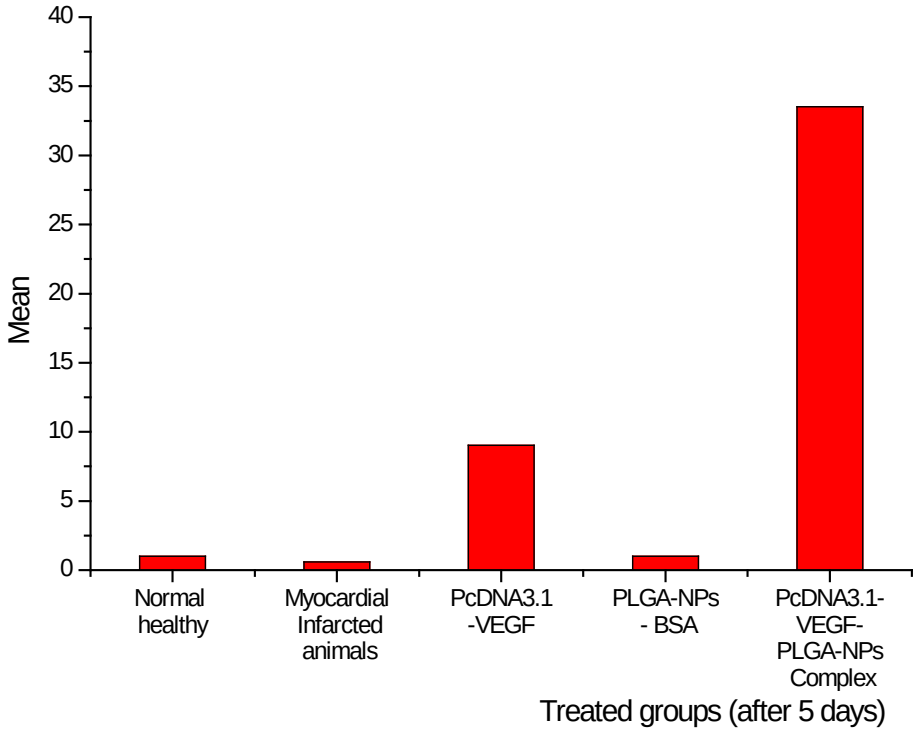


Figure 22: Comparison between fold expression in different treatments after 5 days in relation to normal healthy and myocardial infarcted animals.

The following Table shows comparison between fold expression in different treatments after 7 days in relation to normal healthy and myocardial infarcted animals. There was statistical significant difference between normal healthy and other groups (P1 < 0.05), between myocardial infarcted and other groups (P2 < 0.05), between PcDNA3.1-VEGF with treatment PLGA-NPs– BSA and PcDNA3.1-VEGF-PLGA-NPs Complex (P3<0.05) and between PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex (P4< 0.05).

	Normal healthy	**Myocardial Infarcted animals**	**Treated groups (after 7 days)**		
			PcDNA3.1-VEGF	**PLGA-NPs – BSA**	**PcDNA3.1-VEGF-PLGA-NPs Complex**
Min.	**0.76**	**0.48**	**31.34**	**0.91**	**53.45**
Max.	**1.16**	**0.74**	**38.59**	**2.75**	**145.01**
Mean	**1.01**	**0.60**	**34.77**	**1.56**	**91.65**
S.D.	**0.18**	**0.12**	**3.27**	**0.81**	**41.46**
ANOVA	**29.21**				
P	**0.001***				
P1		**0.014***	**0.001***	**0.041***	**0.0001***
P2			**0.001***	**0.021***	**0.001***
P3				**0.001***	**0.001***
P4					**0.0001***

Table (18): Comparison between fold expression in different treatments after 7 days in relation to normal healthy and myocardial infarcted animals

P1 comparison between normal healthy and other groups
P2 comparison between myocardial infarcted
P3 comparison between PcDNA3.1-VEGF and treatment PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex
P4 comparison between PLGA-NPs – BSA and PcDNA3.1-VEGF-PLGA-NPs Complex

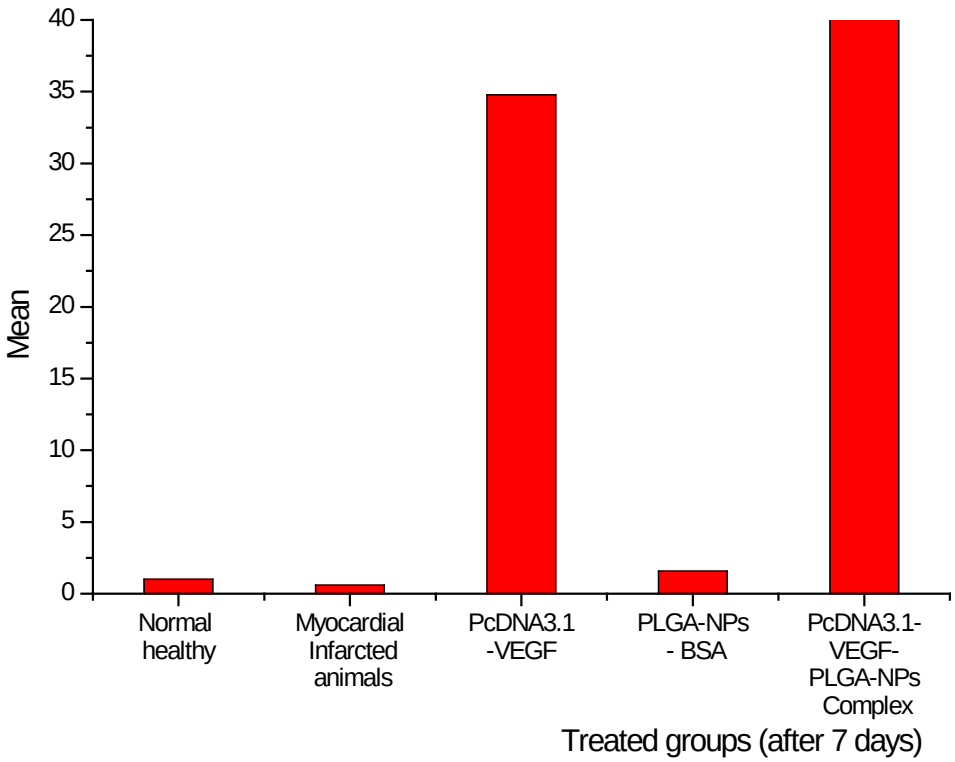


Figure 23: Comparison between fold expression in different treatments after 7 days in relation to normal healthy and myocardial infarcted animals.

The following Table shows effect of duration of treatment on each treatment type. After 3, 5 and 7 days PcDNA3.1-VEGF-PLGA-NPs Complex was higher than PcDNA3.1-VEGF and PLGA-NPs – BSA. There was statistical significant difference between 3 days and 5 days (P1 < 0.05), between 3 days and 7 days (P2< 0.05) and between 5 days and 7 days (P3<0.05).

	Treated groups		
	PcDNA3.1-VEGF	**PLGA-NPs – BSA**	**PcDNA3.1-VEGF-PLGA-NPs Complex**
After 3days			
Min.	**1.79**	**0.59**	**2.04**
Max.	**2.83**	**1.30**	**3.68**
Mean	**2.24**	**0.93**	**2.74**
S.D.	**0.52**	**0.32**	**0.83**
After 5 days			
Min.	**7.57**	**0.48**	**31.34**
Max.	**11.88**	**1.71**	**38.32**
Mean	**9.02**	**1.00**	**33.53**
S.D.	**1.94**	**0.53**	**3.24**
After 7 days			
Min.	**31.34**	**0.91**	**53.45**
Max.	**38.59**	**2.75**	**145.01**
Mean	**34.77**	**1.56**	**91.65**
S.D.	**3.27**	**0.81**	**41.46**
ANOVA	**18.25**	**6.54**	**39.5**
P value	**0.0021***	**0.041***	**0.0001***
P1	**0.002***	**0.654**	**0.001***
P2	**0.001***	**0.042***	**0.001***
P3	**0.0016***	**0.041***	**0.0016***

Table (19): Effect of duration of treatment on each treatment type

P1 comparison between 3 days and 5 days
P2 comparison between 3 days and 7 days
P3 comparison between 5 days and 7 days

4.3. Histopathological finding of heart tissue:

Paraffin sections were stained with haematoxylin and eosin for morphological changes in rat heart tissues for normal and different exposed to treatment. It was noticed the nuclei stained with deep violet color and pink cytoplasm. The white clear color may be indicated to the vacuolated cytoplasm edema at the tissue. . The histopathological changes were illustrated as shown in the following figures:

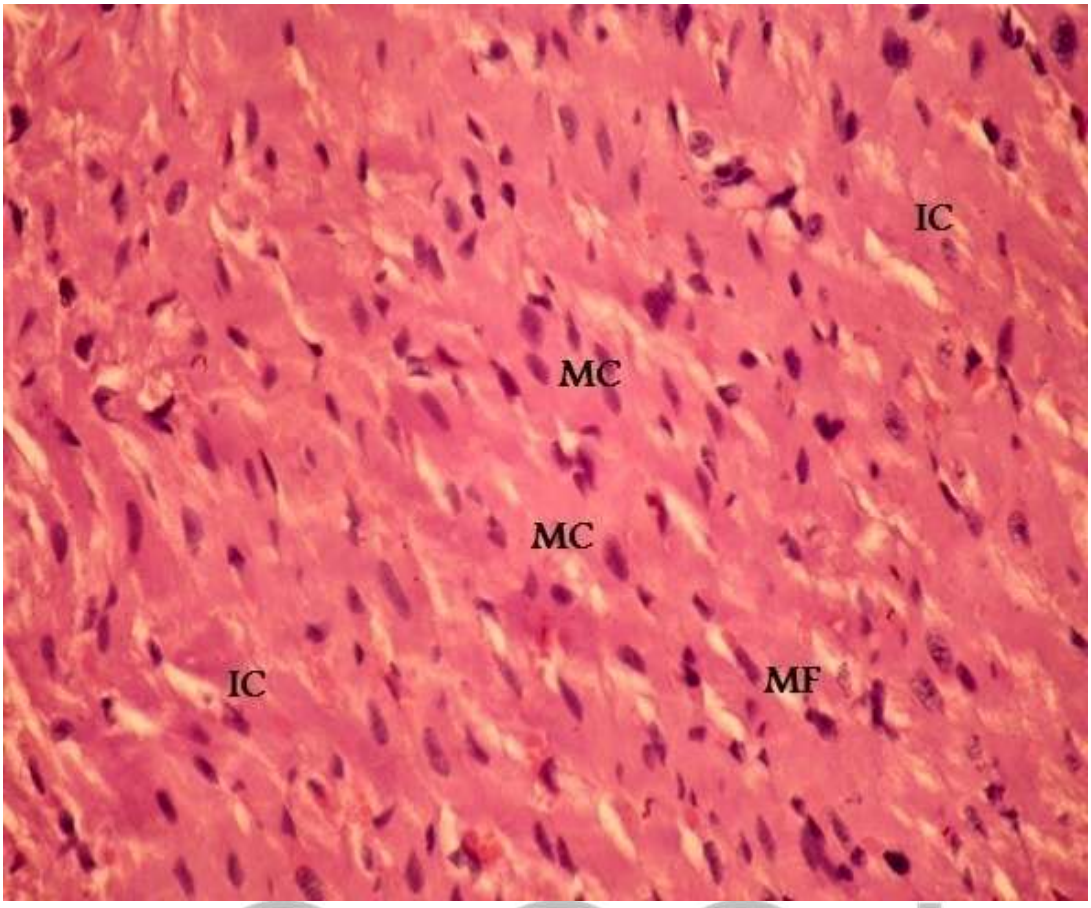


Figure 24: Structure of myocytes of normal rat heart.

The cardiac muscle cells were called myocytes which were consist of Different form of smooth (MC) or skeletal muscle cells (MF) due to placement of nuclei, cross striations, and intercalated disks(IC).

Paraffin section photomicrograph of the normal cardiac muscle tissue indicated that each cardiomyocyte (MC) is attached to its neighboring cell via intercalated disks (IC). These are densely stained ends of the cell that are holding the myofibrils (MF) and the cardiomyocyte's cytoskeleton

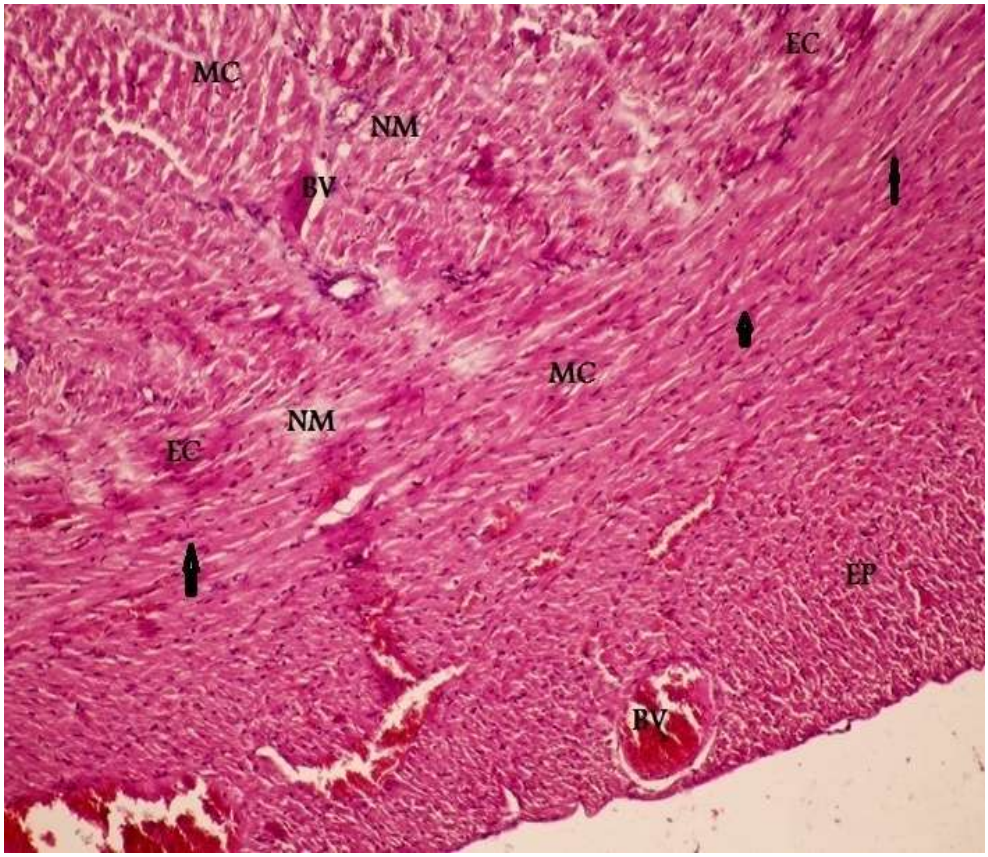


Figure 25: Paraffin section photomicrograph of rat heart, showing the myocardium (MC) layer skeletal muscle cells having hyperchromatic nuclei **(↑)** localized at periphery of cross striations and necrotic myocytes (NM) , there are edema between myocytes , destructive the intercalated disks (EC) and moderate dilated hemorrhage blood vessels (BV) and small foci of inflammatory cellular infiltration. Endocardium layer having a marked dilation of hemorrhage blood vessels (BV) was seen.

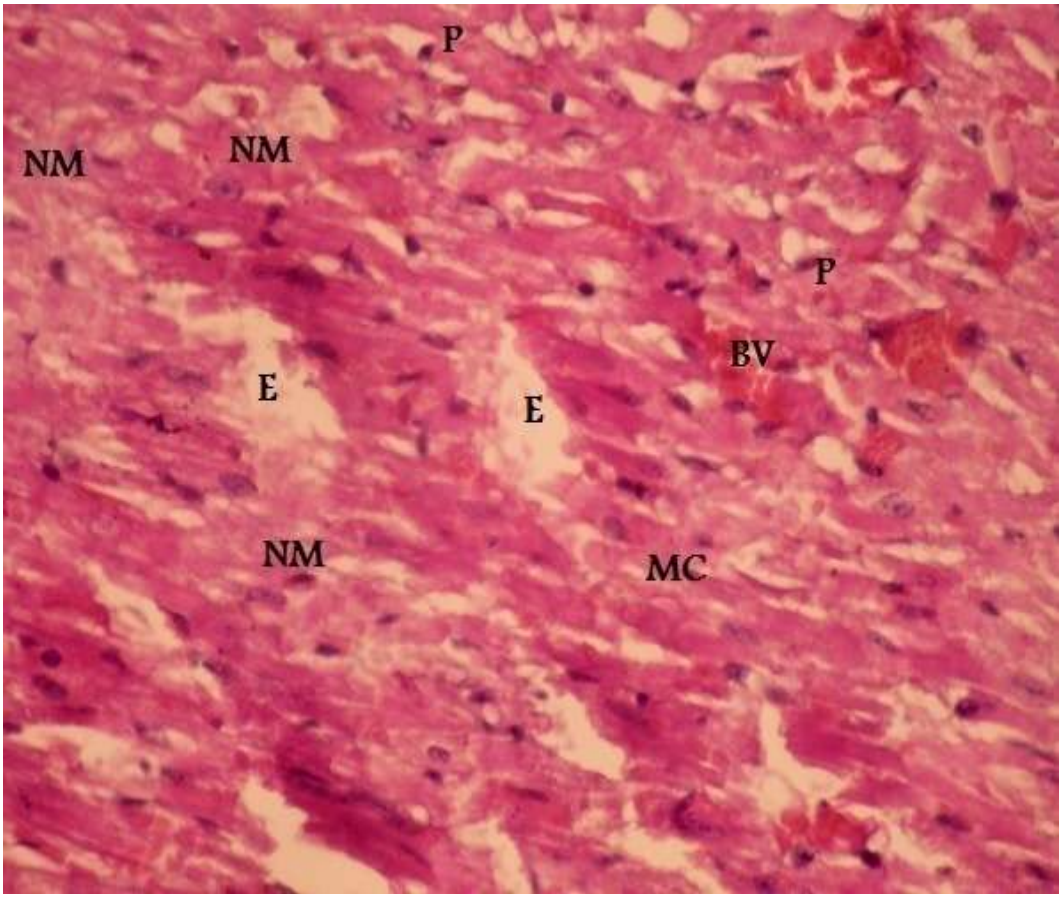


Figure 26: High power Paraffin section photomicrograph of myocardial infarcted rat group heart , Showing necrotic myocardial cell (NM) and pyknotic nuclei cells, increased area of edema between myocytes (E) and destructive the intercalated disks (IC) . The invasive hemorrhage and dilate blood capillaries was seen (BV).

There are highly edema between myocytes, large numbers of destructive intercalated disks (EC) and severe dilated hemorrhage blood vessels (BV) and large foci of inflammatory cellular infiltration. The endocardium layer having a marked hemorrhage blood vessels (BV) indicating myocardial infarction.

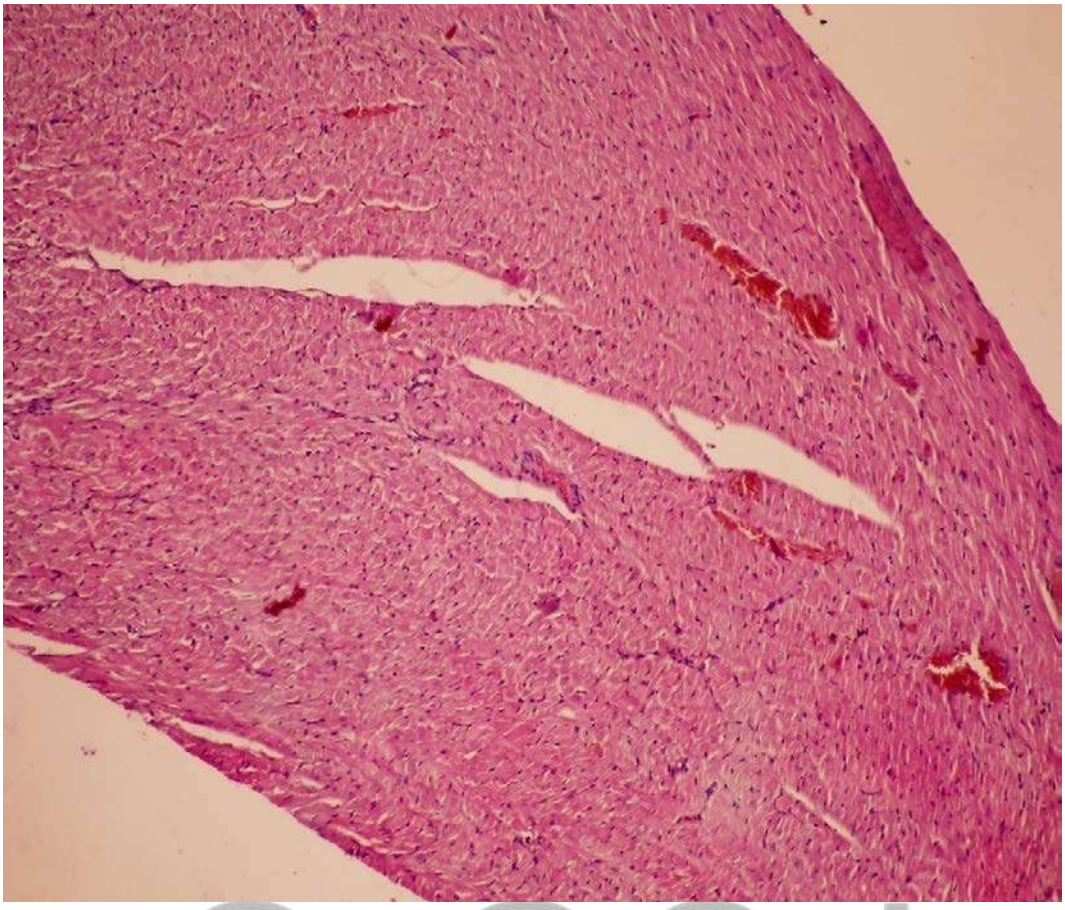


Figure 27: Paraffin section photomicrograph of rat heart for treatment 1 rat group, showing that the myocardium layer having hypertrophy nuclei and area of necrotic myocytes, marked edema between unstriated myocytes, and destructive the intercalated disks with disorganized skeletal fiber and marked dilated hemorrhage blood vessels .The endocardium layer having moderate dilation of hemorrhage blood vessels and infiltrating lymphocytes indicating the effect of therapeutic material.

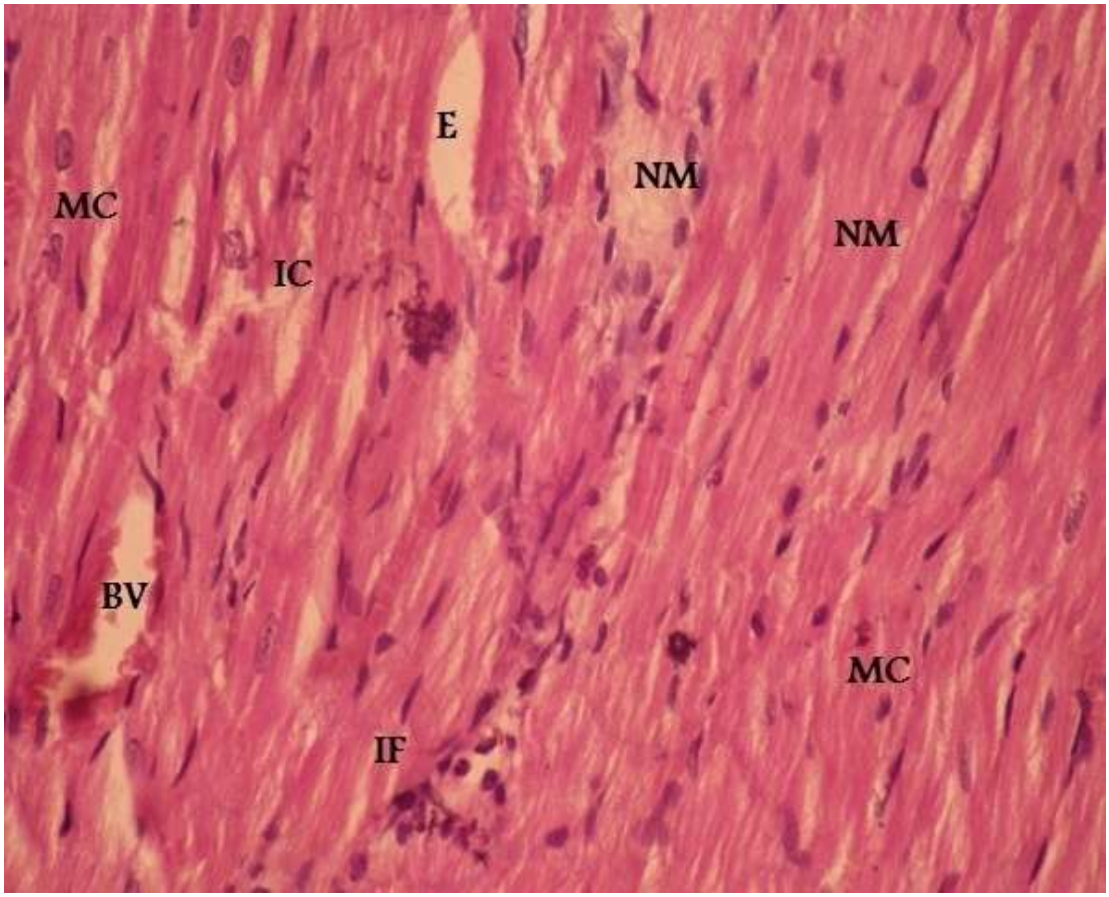


Figure 28: High power of the previous figure for treatment 1 rat group, showing some healthy myocytes with promrnten nuclei (MC), few necrotic myocardial cell (NM), marked edema between myocytes (E) and mild between destructive intercalated disks (IC). Infiltrating lymphocytes (IF) among the myocytes bundles and hemorrhage and mild dilated blood vessels was seen (BV).

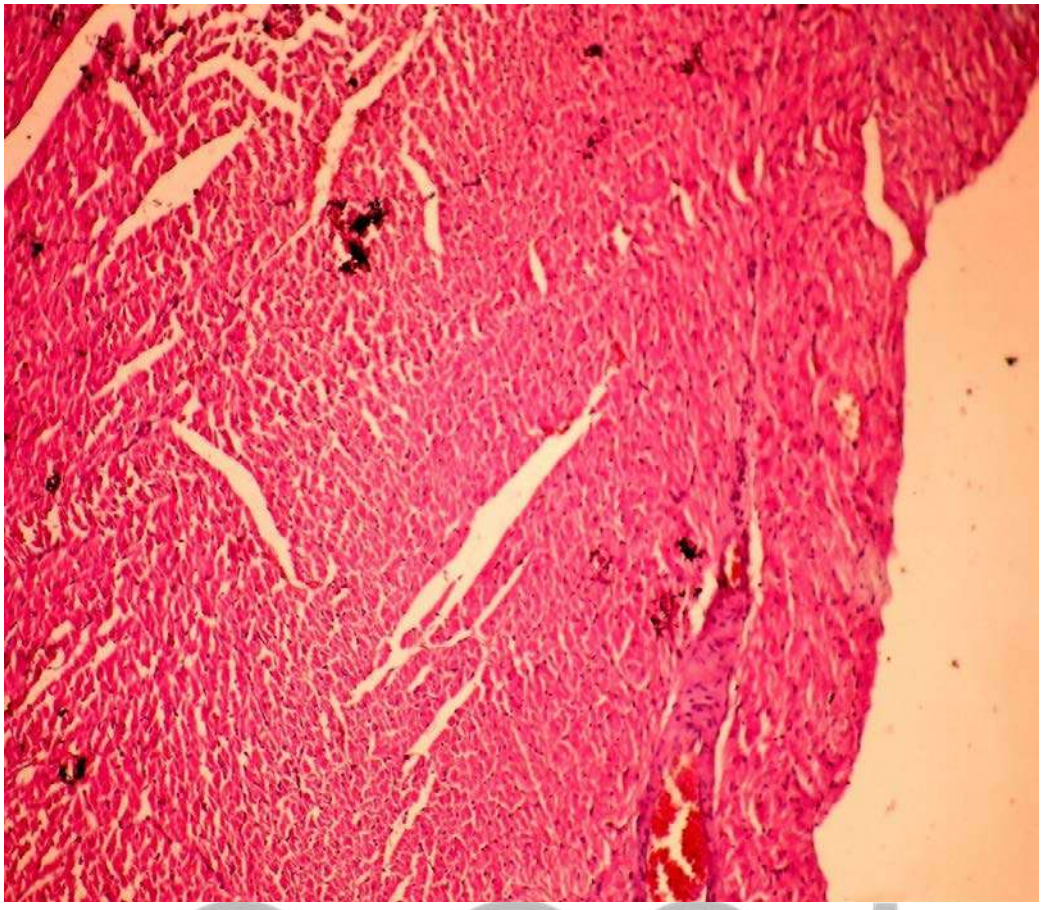


Figure 29: Paraffin section photomicrograph of rat heart of treatment 2 rat group, showing marked edema between myocytes and dense destructive the intercalated disks with unstriated myocytes and skeletal fiber and dilated hemorrhage blood vessels. There are disarray myofibrils foci of necrotic cardicomyocytes as well as area of eosinophilic cytoplasm.

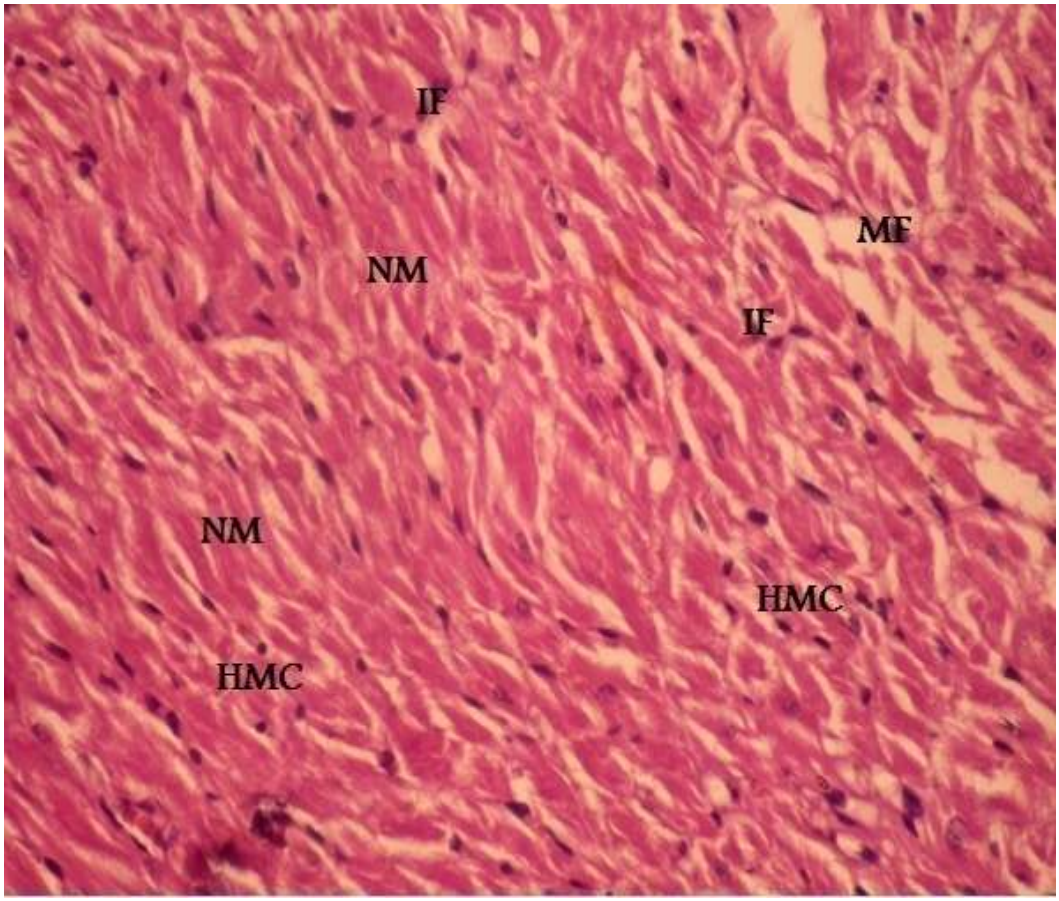


Figure 30: High power of the previous figure for treatment 2 rat group, showing unstriated myocytes having hypertrophy nuclei (HMC) and increased necrotic myocytes (NM), and destructive the intercalated disks (IC), there are area of disorganized skeletal fiber (MF).

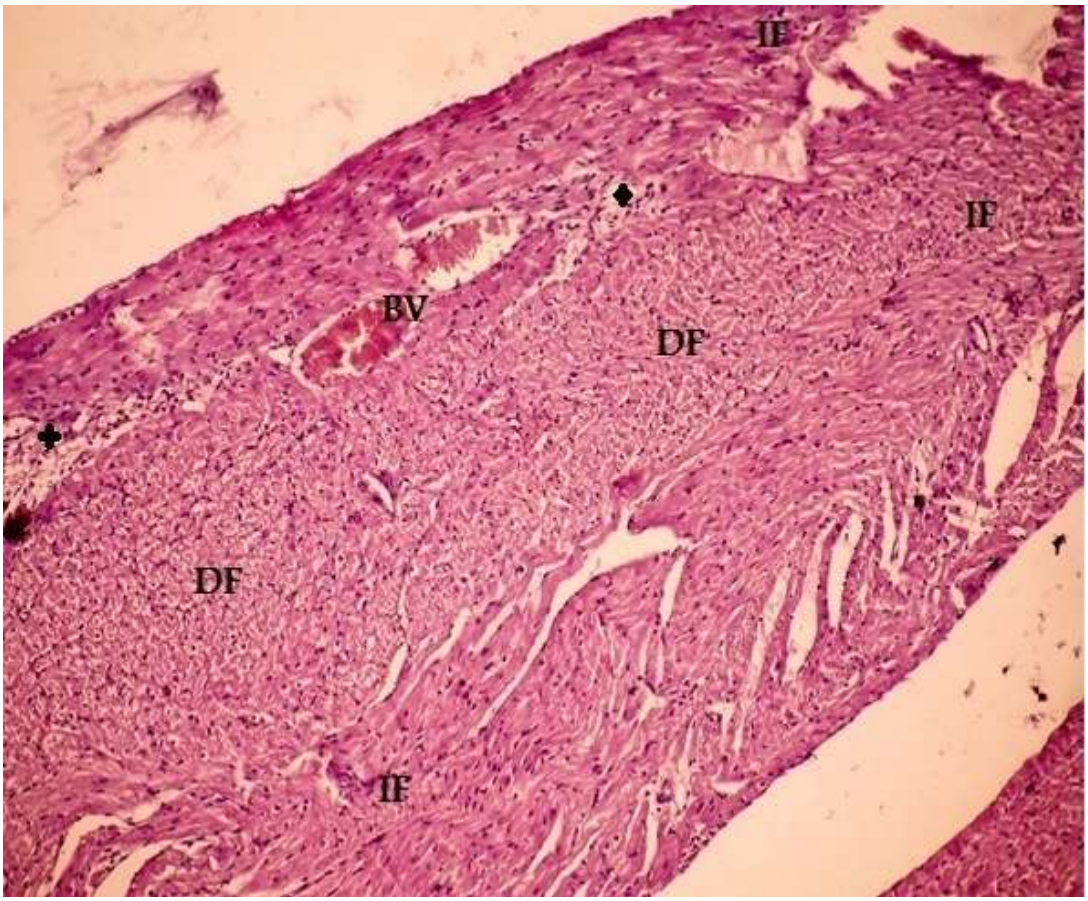


Figure 31: photomicrograph of treatment 3 rat group heart, Showing disorganized of cardiomyocytes, degenerated muscle fibers (DF)with loss of striations dense and different in size of nuclei , area of autolysis muscle fibers (*) with pyknotic nuclei, marked dilation and congested blood vessels (BV) was seen, more infiltrating lymphocytes (IF) and the edema distributed along the tissue.

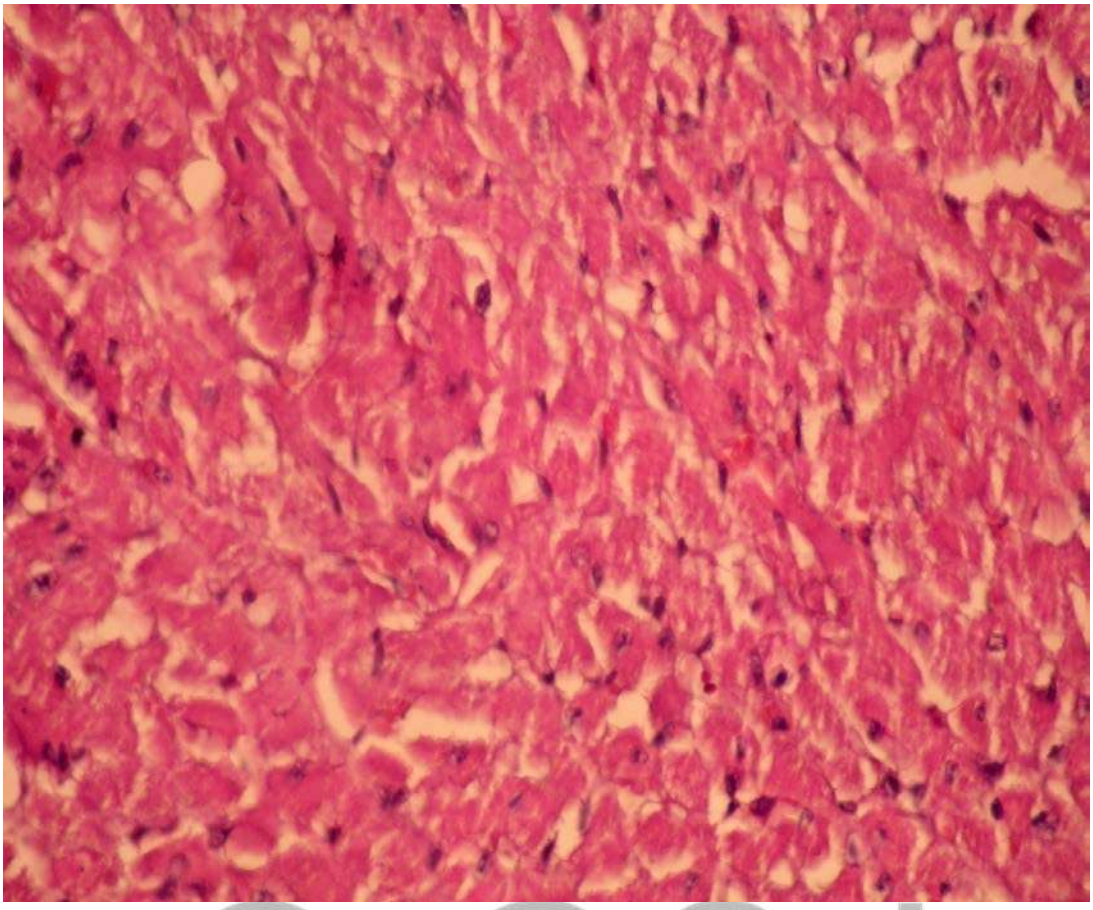


Figure 32: High power of the previous figure for treatment 3 rat group, showing higher healthy myocytes with nuclei (MC), very few necrotic myocardial cell(NM), diminished edema between myocytes (E) and less between destructive intercalated disks (IC) . Infiltrating lymphocytes (IF) among the myocytes bundles and hemorrhage and mild dilated blood vessels was seen (BV).

5-Discussion

Acute myocardial infarction occurance takes place upon occlusion of one of the coronary Vessels, due to atherosclerotic plaque, which results in an ischaemic region of myocardium which, if reperfused, leads to production of lasting tissue damage. Initially, MI leads to production of an inflammatory response and extensive ischaemic death of cardiomyocytes in infracted region, which results in a partial loss of ventricular function. If the affected area is expansive and transmural, alterations in the myocardium occur, this phenomenon is called ventricular remodelling. [68]

Nanotechnology is defined as the understanding and control of matter at dimensions between 1 and 100 nm, also it is a branch of applied Science and technology dealing with the development of devices and dosage forms, this investigates new paths in diagnosis, prevention and treatment of various diseases. Therapeutic agent delivery or active compounds at the desired sites of action are enhanced by nano-sized substances in order to overcome short half-life, low solubility and to reduce toxicity. [69, 70]

Gene therapy is a useful technique using genes that prevent or recover diseases, this allow treating of disorders by inserting a gene in cell of patients instead of drugs. This takes place by replacement of mutant gene causing disease with healthy genes or by inactivation of the mutant gene and introduction of new genes into cells for protection against diseases. [71]

Gene therapy treats myocardial infarction and critical limb ischemia and therapeutic angiogenesis, is done by inducing exogenous administration of genes which are used for treatment for patients who cannot use surgical intervention for treatment or revascularization angioplasty. Gene transfering of angiogenic growth factors includes vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) has resulting in angiogenesis enhancement and functional recovering of ischemic tissues in an animal model. [72]

Non-viral approaches began with simply delivering naked plasmid DNA, but uptake of DNA by cells needs improvement for delivery efficiently. Thus, non-viral delivery systems of genes have employed synthetic chemical vectors to deliver the genes of interest. Synthetic chemical vectors composed of biodegradable materials could avoid the safety issues of viral vectors. In addition, they are easier in manufacturing, purifying, chemically modifying, and can be scaled-up more than that of viral vectors. [73]

Cationic substances which are polymers, lipids, and liposomes can be commonly used as systems for non-viral gene transfer. Condensation of plasmid DNA carrying negative charge with positively charged cationic polymers or lipids can reduce plasmids size several thousand base pairs to

nanoparticle size of 100–200 nm in diameter. This help in protecting plasmid DNA against extracellular degradation and increasing of cellular plasmid DNA uptake via charge interactions. Therefore, the use of cationic substances can improve gene delivery efficiency compared with naked plasmid delivery. Charged polymer-DNA complexes carrying positive charges enter cytoplasm and bind to cell membrane carrying negative charge and endocytosis occurs. [74]

Human VEGF family consists of 5 related glycoproteins VEGFA, VEGFB, VEGFC, VEGFD, and PIGF (Placental Growth Factor) which are secreted from homodimers and interact with family of 3 receptors: VEGFR1, VEGFR2 and alsoVEGFR3. [75]

VEGF role in forming new vasculature it is important to identify different processes in vessel formation. Vasculogenesis takes place during mammalian embryo development. This takes place by forming the *de novo* vessels by differentiation of angioblasts into ECs. During angiogenesis Sprouting is the subsequent process that ensuring expansion of the vessel network. Arteriogenesis occurs by covering of EC channels with pericytes or vascular smooth muscle cells. Many mechanisms can occur, which are intussusception of pre-existing vessels or recruitment of bone marrow derived cells. All of that indicates that VEGF is present and has a critical and an important role. [76]

PLGA is biodegradable aliphatic polyester prepared by condensation of biological monomers, lactic acid and glycolic acid. They are biodegradable, biocompatible and FDA approved for biomedical applications, so PLGA is used in drug delivery systems for many therapeutic agents as in chemotherapy, antibiotics, and antioxidant drugs. [77, 78]

Nanoparticles and microparticles formulated using PLGA polymers are recently being investigated as a new delivery system for genes due to their sustained release characteristics, biocompatibility and biodegradability, and they are able for protecting DNA from endolysosomal degradation. Although PLGA nanoparticles investigation occurs for drug and protein delivery, but their application as a vector for gene expression is novel. [79]

Nanoparticles for PLGA preparation takes place by double solvent evaporation method. Polymer was dissolved in organic phase (4ml) then, sonicated with addition of IAP to make primary emulsion. For the formation of secondary emulsion 16 ml of EAP was added drop wise manner into the primary emulsion during sonication of primary emulsion. Then the secondary emulsion was kept in magnetic stirrer for overnight for excess DCM to evaporate. Separation of particles was done by centrifugating at 15000 rpm for 20 mins. Separated particles washing twice with ice cold MQ water took place then lyophilization of particles was made to obtain dry particles and storage was at -20^{o}C for further use. Similarly, this was done to obtain PLGA – NPs loaded on BSA, the same methodology was followed in case of pcDNA3.1- VEGF loaded particle formation to form Gene transfer complex (Polyplex), but

10ml of EAP was used instead of 16ml, 250 ul of PcDNA3.1-VEGF were added and there was no use of sucrose and sodium bicarbonate in IAP. [80]

Sample 1 (first sample) was the normal sample in which there was no IAP (BSA/PcDNA3.1-VEGF), measuring of the particle size was done. When BSA was added to the particles, the size was decreased. [80]

Nanoparticulate gene delivery, which is based on poly (lactic-co-glycolic acid) (PLGA) polymer, was studied many years ago. Many works has been done to detect biocompatible and biodegradable polymers used in drug delivery. PLGA polymer advantage is that it is well characterized and commercially used for drug delivery systems (Allemann and Leroux, 1999). PLGA polymer is biocompatible, biodegradable. Polymeric nanoparticles are widely used as pharmaceutical dosage form of proteins and peptides. Many methods are recently applied in preparing polymeric nanoparticles, such as emulsification–evaporation method. Biodegradable polyesters, as poly (lactic-co-glycolic acid) (PLGA), has been extensively studied for a wide variety of pharmaceutical and biomedical applications. Biodegradable polyester family is one of the few synthetic biodegradable polymers in which biodegradability can be controlled, excellent in biocompatibility, and is highly safe. Among these polyesters PLGA plays a great role in drug delivery system. [80]

Poly (lactic-co-glycolic acid) have also been called poly (lactide-co-glycolide), according to the nomenclature system based on the source of the polymer. Although the name was used in many references in the past, a recent trend is to follow the nomenclature system of the International Union of Pure and Applied Chemistry (IUPAC) that is based on the repeating unit structure. PLGA can be degraded into non-toxic substances and removed from the human body [81]

Biodegradable poly (lactic-*co*-glycolic acid) (PLGA) polymers show interesting properties for biotechnology through their biocompatibility and their authorization by the Food and Drug Administration (FDA) for drug delivery. Various polymeric drug delivery systems like nanoparticles have been developed using these polymers for the delivery of a variety of drugs (Jain, 2000). However, the technology processes often use organic solvents to dissolve the water-insoluble PLGA. [81]

Biodegradable particles preparation using poly (lactide-co-glycolide) (PLGA) polymer have generated considerable interest in recent years for their use as a delivery vehicle for various pharmaceutical agents. According to Perrin and English, (1997) these polymers are the most common biodegradable polymer used for the controlled delivery of drugs due to its early use and approval as a compatible biomaterial in humans. [82] Lewis, (1990) reported that, by variation in the molecular

weight and also in lactide /glycolide ratio, the degradation time of PLGA and the release kinetics of the active agent can be controlled. [83]

The multiple emulsion-solvent evaporation technique being used for preparation of PLGA nanoparticles is believed to produce heterogeneous size distribution. Formulation factors and characteristics for nanoparticles play a key role in biological applications like drug delivery systems. The main factor influencing the transfection and cellular uptake is the nanoparticles size. [83]

Lemoine *et al.*, (1996) reported that, biodegradable colloidal particles are the possible means of delivering drugs and genes according to several routes of administration. The use of particles prepared from polyesters such as PLGA, is due to they are biocompatible and resorbable through normal bioprocesses in the body. [84]

Encapsulating peptide or protein by PLGA- NPs takes place by three methods which are: water–oil–water (w/o/w) emulsion technique, phase separation methods and spray drying (Freitas, 2005). Peptides or proteins dispersion in an organic solution of PLGA occurs or processing takes place in an aqueous solution of water-in-oil (w/o) emulsion. [85]

According to Niidome and Huang (2002), delivery of plasmid DNA using physical methods resulting in low sustained expression *in vivo*, due to poor uptake which is caused by many factors such as degradation and also clearance. [86]

Herweijer and Wolff (2003) reported that, physical methods such as ultrasound and hydrodynamic injection show improvement for enhancing cellular DNA uptake by altering permeability of the cell. Intrinsic cellular processes are involved due to plasmid uptake, after delivery of gene to the nucleus, expression occurs at a time scale of days to weeks or months. [87] Beginning in the 1980s, many groups demonstrated that intramuscular injection of plasmid DNA led to its transcription in myocytes resulting in encoded protein secretion. [88]

7-Conclusions

Non-viral gene delivery systems using plasmid DNA vectors are showing great promise not only for therapeutic purposes but also in treatment of many diseases including cardiac diseases mainly Myocardial infarction in animal model. Gene transfer and cell targeting with therapeutic genes, plays an important role in gene therapy and repairing the defective organ cells as in myocardium destruction due to myocardial infarction. Non-viral gene delivery is a safe and an accurate way to help in developing a therapeutic action, so it will be the most used way in gene therapy to deliver desired genes to target organ and tissues.

The development of targeted PLGA nanoparticles carrier to a plasmid carrying genes such as VEGF has generated great benefit in targeting process and in gene expression in cardiac myocytes. Targeted PLGA nanoparticles showed effective results in preclinical studies, and cardiac therapy using non-viral gene therapy in which they are used as gene carriers during targeting process. PLGA polymer showed to be an excellent delivery carrier for gene therapy, due to its biocompatibility and biodegradability.

Polymer nanoparticles enhance development of gene therapy for different diseases including cardiac diseases. NPs can be great benefit in non-viral gene delivery methods. The gene targeting using biocompatible PLGA-NPs will result in patient treatment of various diseases as cardiac diseases including myocardial infarction.

The gene transfer complex (Polyplex) "PcDNA3.1-VEGF –PLGA-NPs" in non-viral gene delivery showed the highest expression level in mammalian myocytes, which indicated that it is the mostly effective therapeutic material more than that PcDNA3.1-VEGF and PLGA-NPs-BSA and so promised to be the an angiogenic substance to obtain a recovered myocardium and to improve cardiac function and efficiency.

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Conflict of Interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sectors.

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