A Short Term Training on Animal Handling Techniques

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Contents

CHAPTER 1

Abbreviations

Abbreviations

- **ADP: -** Adenosine Di-Phosphate
- **BUAV: -** British Union for the Abolition of Vivisection
- **cm: -** centimeter
- **CPCSEA:** - Committee for the Purpose of Control and Supervision of Experiments on Animals
- **DNA: -** Deoxyribonucleic Acid
- **GLUT2: -** Glucose transporter 2
- **m: -** meter
- **PETA: -** People for the Ethical Treatment of Animals
- **UK: -** United Kingdom
- **USA: -** United States of America

CHAPTER 2

Introduction

Research is a systematic investigative process to gather new knowledge or revises the previous knowledge over a particular topic or material concerned for establishing new facts and reach new conclusions.

Research is essential for the development of science. The urge for knowledge gives birth to research. It is an on-going process that started from the dawn of civilization and is the primary pillar of the existing civilization. Some people consider research as a movement for known from unknown. It is actually a voyage of discovery. According to Martin Shuttle Worth, "In the broadest sense of the word, the definition of research includes any gathering of data, information and facts for the advancement of knowledge." In other words, by Creswell, "Research is a process of steps used to collect and analyse information to increase our understanding of a topic or issue." There are three steps of research, like, defining a question, obtaining data to answer the question and interpreting an answer to the question. Next step is to investigate in all possible ways to target the question. The data collected is then analysed systematically.

The purpose of research is to discover answers to questions through the answers of scientific procedures. The main aim of research is to find out the truth that is hidden and not yet been discovered.

Research with laboratory animals: Animal testing, alias animal experimentation or animal research is the use of non-human animals to observe (and control) the factors that affect the behaviour or biology of the system under study. It differs from field studies as this form of in vivo testing is conducted in the animal laboratories and not in the natural habitat of the animal in concern. Experimental research with animals is usually conducted in universities, medical schools, pharmaceutical companies, defence establishments and commercial facilities that provide animal-testing services to industry. [1] The focus of animal research is dynamic. It could be solely upon the animal behaviour and development or could be more of an applied research performed in a series of experiments to seek the cure of a disease. Examples of applied research include testing disease treatments, breeding, defence research and toxicology, including cosmetics testing. [2] Most animals are euthanized after being used in an experiment. [3] Sources of laboratory animals vary between countries and species; most animals are purpose-bred, while a minority are caught in the wild or supplied by dealers who

obtain them from auctions and pounds. [4][5][6] Supporters of the use of animals in experiments, such as the British Royal Society, argue that virtually every medical achievement in the 20th century relied on the use of animals in some way. [7] The Institute for Laboratory Animal Research of the United States National Academy of Sciences has argued that animal research cannot be replaced by even sophisticated computer models, which are unable to deal with the extremely complex interactions between molecules, cells, tissues, organs, organisms and the environment. [8] Funnily, animals used in the research laboratories are classified into big animals and small animals apart from invertebrates. Most common non-human lab animals are rats, mice, Guinea pigs, rabbits and frogs. Cats, dogs and monkeys are however used in some experiments. It is undoubtedly said that the usage of animals for research purposes are absolutely necessary, however, the needless employment of animals in unworthy realms is a matter of concern. Animal rights organizations—such as PETA and BUAV—question the need for and legitimacy of animal testing, arguing that it is cruel and poorly regulated, that medical progress is actually held back by misleading animal models that cannot reliably predict effects in humans, that some of the tests are out-dated, that the costs outweigh the benefits, or that animals have the intrinsic right not to be used or harmed in experimentation. [9][10][11][12][13][14] However, this strictly does not imply that the laboratories should be barred from their measured animal usage. There are three R's in the field of animal research first described by W.M.S. Russell and R.L. Burch in 1959. [15] The 3R's state: 1. Replacement - refers to the preferred use of non-animal methods over animal methods whenever it is possible to achieve the same scientific aims. These methods include computer modelling. [16] 2. Reduction - refers to methods that enable researchers to obtain comparable levels of information from fewer animals, or to obtain more information from the same number of animals. 3. Refinement - refers to methods that alleviate or minimize potential pain, suffering or distress, and enhance animal welfare for the animals used. These methods include non-invasive techniques. [16] The 3R's have a broader scope than simply encouraging alternatives to animal testing, but aim to improve animal welfare and scientific quality where the use of animals cannot be avoided. These 3R's are now implemented in many testing establishments worldwide and have been adopted by various pieces of legislation and regulations. [17] Improper usage should be undubiously prohibited and institutions surpassing such actions should be forbidden from doing so. Dignitaries governing such action should spread wide awareness of the misconception brimming out of

foolish hearts about unnecessary animal killing in the labs and should stress on the fact that lab researchers are sufficiently careful about animal usage and handling.

There are four main reasons why animals are used in research:

- **Advance scientific understanding**: Biological research on animals helps us to understand how living things work, and apply that understanding in the benefit of both man and animals. Many cell processes are the same in all the animals like breathing, digestion etc., and many biological systems are alike, like immune systems, nervous systems etc. Thus, animal research can lead to understand what happens to the body. The body's anatomy and functions can be traced to scientific findings from animal research.

- **As models to study diseases**: Humans and animals share hundreds of diseases, and so animals can act as a model for the studies of human illness. Studying disease mechanisms in animals leads to the development of new technologies and medicines that can help both animals and humans. While contributing to our understanding of diseases, animal models enable researchers to explore potential therapies in ways which would be impossible in humans. Studying disease mechanisms in animal models leads directly to the development of new technologies and medicines that benefit both humans and animals. Animals which are altered to create models of disease are known as induced models.

- **To develop and test potential forms of treatment**: Animals are used to test and develop potential therapies. Diagnostic tools are safe and effective because they are tested on animals successfully. Many surgical techniques rely on the methods and equipment that are developed using animals.Humans share at least 90% of their genes with every other mammal. Over 95% of the genes are homologous between mice and humans.The short life span of research animals allows scientists to study them throughout the entire life cycle—and even through several generations—within a short period of time.

- **To protect the safety of animal, people and the environment**:New medicines require testing to ensure both the beneficial and harmful effects on a whole organism. Legally and ethically it must be tested in a suitable animal model before clinical trial on humans. Testing on animals also serves to protect people and environment from the harmful

effects of chemicals. All the chemicals for commercial and personal use must be tested so that their effect on people and animals can be understood. [5]

At least 100 million animals,including mice, rats, frogs, dogs, cats, rabbits, hamsters, guinea pigs, monkeys, fish, and birds- are used every year for research purpose in the multibillion dollar research industry that includes university, pharmaceutical and diagnostic laboratories, and many others. The following chart shows which animals were used for research, testing, and educational purposes in the USA in 2010: [6]

Animal	Number
Mice, Rats, Fish, and Birds	25 million
Guinea Pigs	213,029
Rabbits	210,172
Hamsters	145,895
Nonhuman primates	71,317
Dogs	64,930
Pigs	53,260
Other Farm Animals	38,008
Cats	21,578
Sheep	13,271
Marine Mammals	126
Other species	303,107

Rabbits (*Oryctolagus cuniculus*) are small mammals in the family of Leporidae of the order Lagomorpha. Newly arrived rabbits for the experiment is at first pre-treated for several weeks, and are examined for the most common diseases. Rabbits can be easily affected with contagious diseases like scabies, which can lead to pneumonia. Adult rabbits should be kept in individual mesh cages (0.90m x 0.90m x 0.45m) hung at a height of 0.8 cm from the ground so that the excrement can fall into tray which should be placed underneath the cage. 12 to 14 hours of light is necessary for the proper biorhythm and rabbits should be properly and routinely observed for food consumption and facial characteristics. The diet of an adult rabbit should be consisting of hay, water and fresh vegetables. Water should be available

throughout the whole of the day and should be changed as frequently as possible, like, every day, because dehydration is a very common and serious condition in rabbits and should be attended to immediately and carefully. [7]

Guinea pig (***Caviaporcellus***) is a species of rodent belonging to the family Canidae and the genus Cavia. It has a robust body with short limbs, large head and eyes, and short ears. It has feet with hairless soles and with sharp claws. It has four toes on the forefeet and three on the hind. Guinea pigs are vegetarian and do not require drinking water if they are supplied with sufficiently moist food. They breed throughout the year in captivity. Females bear up to 13 young per litter (4 is average). Gestation takes 68 days. For one guinea pig, a minimum of 7.5 sq. feet cage is required, but more is better, generally 30" x 36" is a good size for one guinea pig. [8]

Rats are the first mammalian species which are bred and used for scientific research in research laboratories. A laboratory rat of the species ***Rattus norvegicus*** (brown rat) is bred and kept for laboratory research. The albino laboratory rat which has red eyes with white fur, are very frequently used for research as a model organism in a variety of fields. Rats are omnivores. As rats are developed as laboratory species, rats have been used to answer a wide range of basic science questions in the fields of pharmacology, immunology, psychology, toxicology etc. [9]

Mice are small mammals of the order Rodentia which is bred and used for scientific research in genetics, psychology and medicine. Mice are mainly herbivores and eat almost all kind of fruits and grains. In laboratory, mice are fed with nutritionally complete commercial pelleted mouse diet. They are usually maintained at 17.8 to 26.1°C and 30% to 70% humidity. Cages provided should have enough space; a female mouse and her litter should have a minimum of 51 sq. inches of floor area and cage height of 7 inches. Mice prefer socializing, and staying in groups, so it is better to keep them in groups, better to house 4 to 5 mice in a cage together, but not more than 9. [10]

CHAPTER

3

AIM & OBJECTIVES

AIM:

The main aim of the present study is to understand the different types of animals used in research work. The different handling techniques are learned in rats.

OBJECTIVES:

- To study the maintenance of laboratory animals.
- To study holding a rat.
- To study the feeding of a rat.
- To study the feeding habit of animals.
- To study various techniques.
- To study Retro-orbital blood collection from rat.
- To study rat dissection and identification of its various organs.

CHAPTER

4

Materials & Methods

MATERIALS

- **Animals used-**Wistar albino rats weighing 150-200 gm, Mice weighing 30-35 gm.
- **Chemicals –** Normal saline.
- **Instruments used -** Weight machine, Glucometer, Nerve Conduction Velocity machine, UV spectrophotometer.
- **Other requirements –** Micro pipettes, tips, syringe, feeding cannula.

METHODOLOGY

- Methods learnt in the present study include the following:
- Handling Techniques of animals
- Feeding of Animals
- Development of Diabetes rat models
- Estimation of Blood Glucose of Animals
- Estimation of Nerve conduction velocity in animals
- Blood Collection from Rats
- Rat sacrifice and dissection

CHAPTER

5

Observation

Maintenance of all animals

All rats were maintained according to the CPCSEA guidelines at the animal laboratory of R.G. Kar medical college, Kolkata, after getting clearance from Institutional animal ethics committee from R.G. Kar Medical College, Kolkata. The temperature was maintained between 18°C-21°C and relative humidity between 30%-70%. The animals were maintained in 12-hour light and 12-hour dark cycle in animal house. The animals had given sufficient amount of animals feed and water supply in animal room.

Handling techniques of animals

Rabbit handling

Daily contact with human touch reduces the stress of handling an animal. Gloves should always be worn while handling a rabbit. If they are not properly handled, they can become nervous and can inflict bites or scratches to wound the handler. Rabbits should never be handled by the ears because of a high probability of cervical luxation which may cause death. They should be held by grasping a large fold of loose skin over the shoulders with one hand and either supporting or grasping the rear feet with the other hand. Failing to support or hold onto the rear feet may result in giving chances to them to kick and try to escape, which can cause severe spinal injury or a broken back.

Guinea pig handling

Guinea pigs have delicate bones. It is important to learn how to properly handle a guinea pig, because some guinea pigs tend to jump especially when the handler is returning it to its cage, this can cause broken bones, injury and sometimes death. They should be wrapped with one hand securely around the chest. One front leg should be restrained by placing a finger in front of it. Other hand should be used to support the hind feet and rump. This holding technique is particularly important when to handle a nervous guinea pig which tends to bite when being transported.

Rat handling

Rats are generally docile animals, particularly if they are routinely handled with appropriate techniques. Bites from rats are quite uncommon, and only occurs when they are stressed or in pain. Initially it should be grasped around the shoulders to restrain it; thumb of the handler

should be placed under the rat's mandible to prevent bites, and the rat's hind limbs should be supported with the other hand. Restraint should be firm, but not to firm to impede the animal's respiration.

Mice handling

Mice are generally easy to restrain, but their small size makes them vulnerable to physical injury. Some mice attempt to jump out from the grasp of the handler. Mouse should be grasped by the tail, preferably the proximal third and lifted clear of its cage. Then it should be placed on a surface like a cage top. If a gentle traction is made on the tail, it will grip the cage top and will try to flee away. The scruff can be grasped between the thumb and forefinger while maintaining a grip on the tail. This technique makes a secure and firm grip to be examined and injected safely.

Feeding of animals

There are metal and flexible plastic feeding needles called cannula available in various sizes for rats and mice. Metal gavage needles are easier to use in mice as they cannot bite through the tube, but it can easily damage the oesophagus if the mouse struggles. Flexible plastic gavage needles have less chance to damage the oesophagus but they can bite through them, and it also requires some practice to use effectively.

Mice

At first it should be removed from the cage and firmly restrained in an upright position. Good scruff of skin should be gripped so that its front legs are extended out to the side and the head and neck are immobilized, but it must be ensured that the animal is breathing properly (by seeing whether the chest is moving). Then the gavage needle is inserted into the left side of its mouth and is directed along the hard palate of the mouth to the back of the throat.

Rat

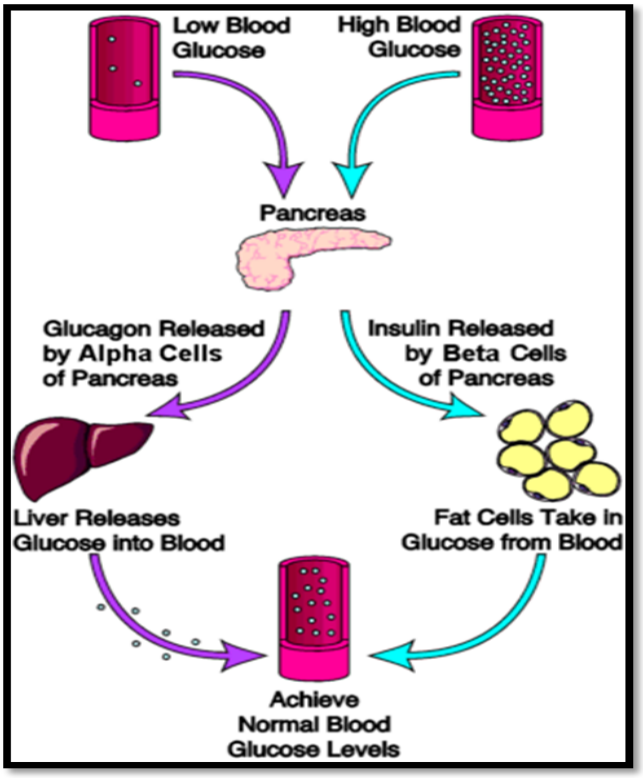
It is at first gently removed from the cage, and then it is restrained using either a v-hold or crossover to immobilize the head and neck of the rat, and then it is fed with cannula, but always it must be made sure that it can breathe freely. If a flexible red rubber feeding tube is used, the rat is then gently restrained by hand or by wrapping with the help of a towel while

they are sitting flat on a table or counter. Gavage needle is inserted into the left side of its mouth and is directed along the hard palate of the mouth to the back of the throat.

Development of diabetes rat model

Principle

Diabetes mellitus is a type of heterogeneous group of disorders of carbohydrate metabolism that leads to defect in insulin secretion. Normally, when the blood glucose level of the body decreases below the threshold level, alpha cells of the pancreas secretes glucagon hormone to regulate the function of the liver to secrete glucose into the blood, to maintain the homoeostasis. Contrary to that, when the blood glucose level increases above the threshold level, it gives signal to the pancreatic beta cells to secrete insulin which restores the normal glucose level of the blood, through the uptake of the fat cells. So usually, in normal human body, the blood glucose level is maintained by two pancreatic hormones. When this equilibrium breaks, it causes metabolic disorder, Diabetes. (Photograph 1) [18]



Photograph 1: Maintenance of blood glucose level in normal condition. [18]

For the sake of experiment, different types of diabetes models of rats are used, like, genetically derived diabetic animal (Zucker fatty acid is used); chemically induced rat (low dose of Alloxan or Streptozotocin adult rats or mice are used) and nutritionally induced diabetes rats are used (high fed rats are used).

In the present study, Streptozotocin-Nicotinamide was used to induce diabetes in rats. Streptozotocin (STZ) is produced by ***Streptomyces achromogenes.***

Streptozotocin causes damage to pancreatic beta cells. It is taken up by the beta cells of the pancreas and thereby causing damage to DNA. The mechanism can be stated such that Streptozotocin in transported into beta cells via glucose transporter **GLUT2** and causes DNA damage, and also passively increases the activity of **poly ADP-ribose polymerase** enzyme to repair DNA. It also inhibits insulin production. DNA damage in turn increases the activity of poly (ADPribose) polymerase (PARP1) enzyme that helps to repair DNA. DNA repair mechanism utilizes NAD^+ and ATP in the cells. This increasing activity of the enzyme leads to the depletion of intracellular NAD+ and ATP, and because of this, the insulin producing beta cells undergo necrosis.

Nicotinamide partially protects the insulin secreting cells against STZ. Nicotinamide (NA) is used to protect against the detrimental effect of Streptozotocin-induced β cells toxicity. Nicotinamide shows protective action by inhibiting the activity of poly ADP-ribose polymerase enzyme. So, the intracellular NAD+ and ATP which were exposed to STZ, is prevented. Nicotinamide also serves as a precursor of NAD+, so, additionally it also increases the intracellular NAD+ levels.Thus NA helps to nullify the damage caused by STZ. Therefore this combined effect mimics the condition encountered in human suffering from Type 2 Diabetes Mellitus.

The severity of diabetes in experimental rats strongly depends on the dosage of STZ and NA given to these animals. [19]

Protocol

Diabetes was induced in rats by streptozotocin (STZ) and Nicotinamide (NA). Intraperitoneal (ip) injection of NA is induced in it (figure 1) at the dosage of 110 mg/Kg in 12-hours fasted rats then and after a period gap of 15 minutes,STZ is induced intravenously (figure 2) at the dosage of 60mg/Kg body weight in 0.1M citrate buffer (pH- 4.5) in the rat through the tail of

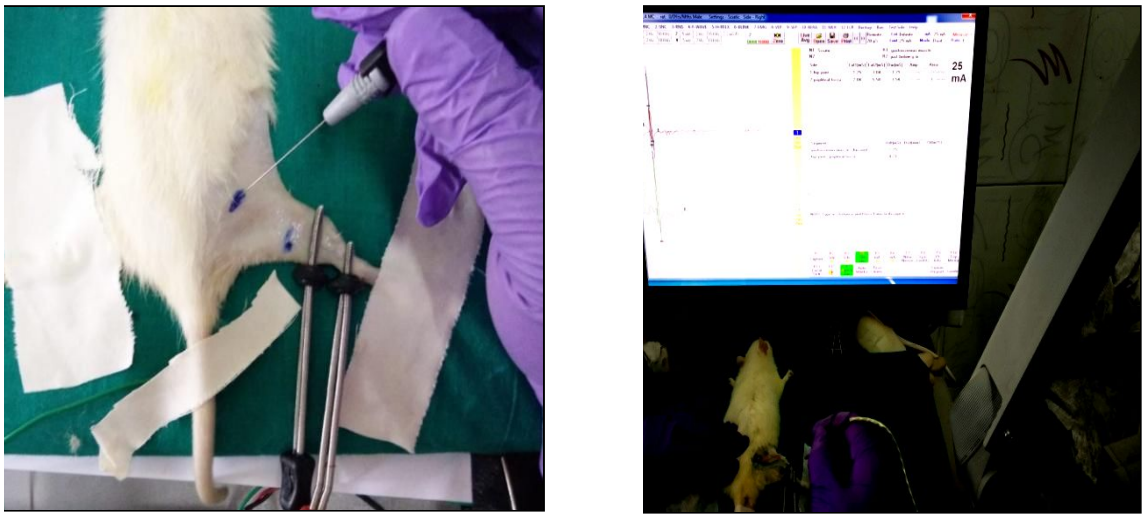
it. Thus, the same process is continued with every rat considered for the experiment. Blood glucose with blood collected from the tail-vein was monitored after 3 days and rats with blood glucose more than 250 mg/dl was considered as the diabetic group. Drug was administered orally after 48 hours.

Estimation of Blood Glucose of Animals:

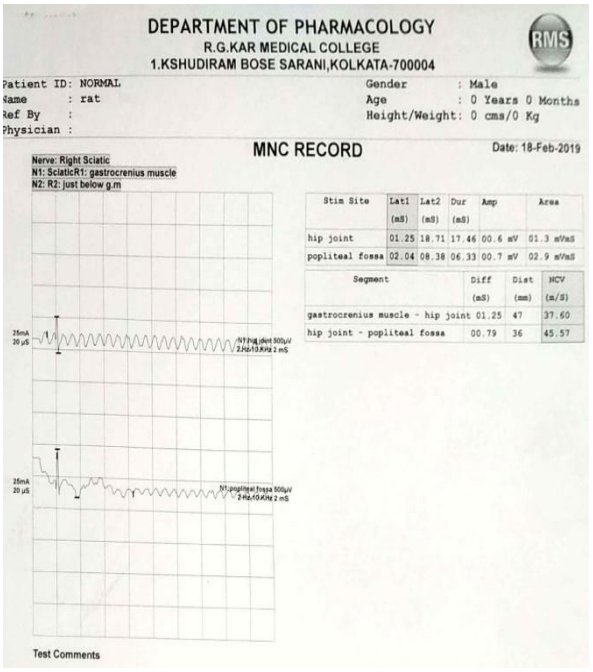
The rat is placed out of the cage on a flat platform. The loose skin behind the ears is grasped by the thumb and forefinger. The tail of the rat is held firmly. The tip of the rat tail is slightly imputed at the farthest tip with a new blade. The tail is massaged and the blood oozing out is placed on the glucose strip of a glucometer. The reading is noted down as the initial blood sugar level.

Motor nerve conduction study:

Electrophysiological assessment was done by motor nerve conduction velocity test of sciatic nerve in the rats at 12 wk and 24 wk. Animals were anaesthetized using thiopentone sodium 45 mg/kg i.p. Thereafter, animals were positioned with maximally straightened hind limbs & necessary trimming and depilation were done for removing fur to reduce noise interference in the reading. Electrophysiological measurements were taken using EMG EP MARK II (Recorder & Medicare System Pvt. Ltd, India). The common reference (ground electrode) was placed on the basis of tail. The sciatic nerve of each experimental animal was stimulated percutaneously with bipolar needle electrodes which were placed at the level of hip joint and popliteal fossa. Recording bipolar needle electrodes were placed in medial gastrocnemius muscle belly as described earlier. [20] Electrical stimulation was square pulse with a high frequency filter 10 kHz & low frequency filter of 1Hz, range 25mA, sensitivity 1mV, time sweep 2ms/div and duration of 0.02ms. The stimulus current intensity was increased gradually up to supramaximal intensity. Then the rat's hind limbs were straightened and the distance between the stimulating electrodes were ascertained with a tape measure to the nearest millimeter. These measurements were inputted into the company software (EMG EP MARK II) and the results of the sciatic nerve were obtained.



Photograph 2: Nerve Conduction Velocity measurement in rats



Photograph 3: Nerve Conduction Velocity in normal rats

The evaluation of peripheral nerve conduction velocity (NCV) showed significant reduction in rats induced with diabetes as compared to normal.

Rat sacrifice and dissection

Sacrifice

None of the end of life option is perfectly pain free, whether chemical or physical methods are used. Cervical separation requires highly experienced handlers. Anaesthetizing the animal before killing it may eliminate consciousness and so reduces the experience of stress and pain. All physical methods should be practiced on dead animals under the supervision of an experienced technician or investigator before attempting to euthanize an animal. All physical methods should confirm death.

Dissection:

The rat is pinned down by placing the ventral side up. The abdominal skin is lifted with forceps and cut through with the scissors. The scissor blade is inserted under the skin. The rat is cut along the midline from pubic region to lower jaw. The skin is freed from underlying connective tissue and muscle. The underlying organs were then visible.

Tissue Antioxidant Study

All animals were sacrificed under deep anesthesia and blood was withdrawn from retro-orbital plexus. The antioxidant parameters like lipid peroxides, superoxide dismutase and reduced glutathione were evaluated in liver, kidney and pancreas tissues.

Lipid peroxidation assay. 0.5 ml of 10% tissue (liver or kidney) homogenate in phosphate buffer was added to 1 ml (or 2mL) of TBARS solution [0.375% w/v thiobarbituric acid, 15% w/v trichloroacetic acid, 0.25 N hydrochloric acid]. The solution was heated in boiling water for 30 min and then cooled. It was then centrifuged at 3000 rpm for 15 min. The absorbance of supernatant measured at 532nm against blank. [21] Amount of malonaldehyde (MDA) was calculated with the help of the standard curve plotted with MDA in respect to the amount of protein present in tissue.

Superoxide dismutase assay. 1.2 ml of sodium pyrophosphate buffer (0.052 mM; pH 8.3), 0.1 ml of 186 μM phenazine methosulphate (PMS), 0.2 ml of 10% tissue (liver or kidney) homogenate in phosphate buffer and 1 ml of distilled water were added to each reaction

mixture. Thereafter 0.3 ml of 300 µM nitroblue tetrazolium (NBT) and 0.2 ml of nicotinamide adenine dinucleotide *i.e.* NADH (780 µM) was added to start the reaction. The reaction mixtures were incubated in dark at room temperature for 90 seconds and reactions were stopped by adding 1 ml of glacial acetic acid. The amount of chromogen formed was measured by recording color intensity at 560 nm. Amount of superoxide dismutase (SOD) was calculated with the help of the standard curve plotted with SOD in respect to the amount of protein present in tissue.[22]

Reduced glutathione level. The method is based on the reduction of 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione to produce a yellow compound. 100 µl of 10% tissue homogenate in phosphate buffer was dissolved in 600 µl of 20 mM EDTA and incubated in ice for 10 min. 500 µl of water, 250 µl of TCA (10% w/v) was added and incubated at room temperature for 5 min. The mixture was then centrifuged at 5000 rpm for 10 min. 2 ml of Tris buffer (0.4M) and 100 µl of DTNB (0.1M) was added to 1 ml of the supernatant and incubated at room temperature for 3 min. Absorbance was measured at 412 nm. Amount of GSH was calculated with the help of the standard curve plotted with GSH in respect to the amount of protein present in tissue. [23]

Concentration of MDA was found to be significantly higher in diabetic rats as compared to the normal rats. On the other hand, SOD activity and GSH level were found to be lower in diabetic rats in comparison to normal control.

CHAPTER

6

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