"DETERMINATION OF MITOCHONDRIAL DIVERSITY PATTERN IN THE NEUTROPHILS OF NORMAL MALES"

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SUBMITTED BY

RINKAL MEHTA

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UNDER THE GUIDANCE OF

Dr. SRIRAM SESHADRI

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DECLARATION

I declare that the thesis entitled **DETERMINATION OF MITOCHONDRIAL DIVERSITY PATTERN IN THE NEUTROPHILS OF NORMAL MALES** has been prepared by me under the guidance of **Dr SRIRAM SESHADRI,** Assistant Professor of **INSTITUTE OF SCIENCE**, **NIRMA UNIVERSITY.** No part of this thesis has formed the basis for the award of any degree or fellowship previously.

RINKAL MEHTA
INSTITUTE OF SCIENCE, NIRMA UNIVERSITY.
SARKHEJ-GANDHINAGAR HIGHWAY, AHMEDABAD-382481, INDIA

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ABSTRACT

ABSTRACT

Among the numerous theories that explain the process of aging, the mitochondrial theory of aging has received the most attention. This theory states that electrons leaking from the ETC (Electron Transfer Chain) reduce molecular oxygen to form superoxide anion radicals, through both enzymatic and non-enzymatic reactions, can cause the generation of other ROS (reactive oxygen species). The ensuing state of oxidative stress results in damage to ETC components and mtDNA (mitochondrial DNA), thus increasing further the production of ROS. Ultimately, this 'vicious cycle' leads to a physiological decline in function, or aging.

Neutrophils, the primary phagocytic cells of the human immune system, have a very short life span after leaving the bone marrow of approximately 24 hours. Thereafter, they die either by intrinsically or extrinsically induced apoptosis. Intrinsically, neutrophil apoptosis appears to be regulated at the level of their mitochondria.

In the present study, age group ranging from 21 to 55 were taken for the blood profiling, serum biochemical parameters and mitochondrial proteins and enzyme activity determination studies of normal healthy men. In the present study, neutrophil has been used as the somatic cell representative.

The results show that the values of blood profiling parameters and serum biochemical levels did not show any trend and was found to be fluctuating within the normal range. Antioxidants assay were performed which revealed a decline in the mitochondrial SOD and Catalase activities with increasing age. The activities of the other mitochondrial enzymes, Succinate dehydrogenase and Malate dehydrogenase have shown variations in the neutrophil.

It can be thus concluded that there is a gradual decline in most of the mitochondrial antioxidant levels and enzymes with increase in age. The same needs to be confirmed with the mitochondrial apoptotic marker enzymes.

The polymorphism found in the mitochondrial enzymes of neutrophil can be further checked at the genetic levels. And the variations obtained can be co-related with mitochondrial dysfunctioning and mitochondrial associated neurodegenerative diseases with age as parameter.

Mitochondria are the primary energy-generating system in most eukaryotic cells.

Mitochondria evolved from a symbiotic relationship between aerobic bacteria and primordial eukaryotic cells (Wallace, 2005). A mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells (Martin et al., 2003). These organelles range from 0.5 to 10 micrometers (μm) in diameter. Mammalian mitochondria are ubiquitous organelles responsible for 90% of ATP production in respiring cells (Mootha et al., 2003).

A mitochondrion contains outer and inner membranes composed of Phospholipid bilayer and Proteins. The two membranes have different properties. Because of this double-membraned organization, there are five distinct compartments within the mitochondrion that carry out specialized functions. These compartments or regions include the outer membrane, the inter-membrane space, the inner membrane, the cristae and matrix. In mammals, they are present in all cell types apart from anucleate red blood cells. Most cells contain very many mitochondria, with the largest numbers (more than 10^4) in those with the highest energy requirements. In extreme cases, for example in humming bird flight muscle, mitochondria can constitute 35% of the cell volume (Rich and Maréchal, 2010).

The number of mitochondria in a cell varies widely by organism and tissue type. Many cells have only a single mitochondrion, whereas others can contain several thousand mitochondria. In neutrophil, the number of mitochondria ranges from 87±10 in inactive state to 600±77 in active state (Marinos et al., 1981).

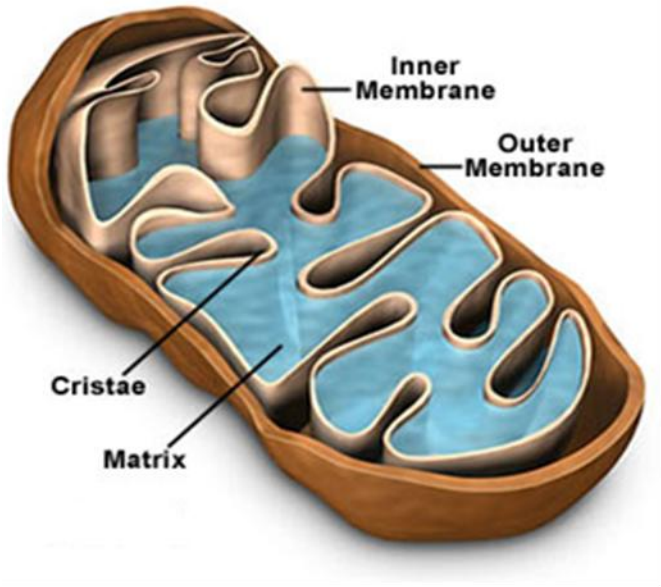
The outer membrane forms the boundary with the cytoplasm. It is permeable to small molecules and has specific proteins to facilitate inward transfer of cytoplasmically synthesized mitochondrial proteins. It is also the site of many other specific biochemical functions, including a set of poorly understood processes that can trigger release of mitochondrial components into the cytoplasm that then induce cellular apoptosis. It contains large numbers of integral proteins called porins. These porins form channels that allow molecules 5000 Daltons or less in molecular weight to freely diffuse from one side of the membrane to the

other (Johnson et al., 1994). The inter-membrane space is the space between the outer membrane and the inner membrane. One protein that is localized to the inter-membrane space in this way is cytochrome C (Green et al., 2006). The inner membrane houses around 1/5 of the total protein in a mitochondrion (Johnson et al., 1994).

There is a membrane potential across the inner membrane formed by the action of the enzymes of the electron transport chain. There are four carriers of electron transport, complexes I to IV, embedded in the lipid bilayer of the inner mitochondrial membrane (IMM). The IMM is usually highly invaginated in order to increase its surface area so that it can house the vast numbers of respiratory chains and ATP synthases that are needed to produce the power that cells and organism require. The inner mitochondrial membrane is compartmentalized into numerous cristae. The matrix is the space enclosed by the inner membrane. It contains about 2/3 of the total protein in mitochondria. The matrix contains a highly-concentrated mixture of hundreds of enzymes, special mitochondrial ribosomes, t-RNA and several copies of the mitochondrial genomic DNA. Of the enzymes, the major functions include oxidation of pyruvate and fatty acids, and the citric acid cycle (Johnson et al., 1994).

The outer and inner membranes of mitochondria differ in their complement of enzymes (Parsons et al., 1966; Beattie, 1968; Schnaitman and Greenawalt, 1968), as well as in chemical composition (Parsons et al., 1967; Smoly et al., 1970). Following are the enzymes of the outer membrane: Kynurine hydroxylase, monoamine oxidase and rotenone insensitive NADH Cytochrome c reductase, Apoptosis regulator Bcl-2 and monoamine oxidase. Adenylate kinase for the inter membrane space, Succinate Cytochrome c reductase and rotenone-sensitive NADH Cytochrome c reductase for the inner mitochondrial membrane and NAD+ malate dehydrogenase as markers for the mitochondrial matrix (Gear, 1970).

Figure 1: Diagram showing mitochondria inner structure
(www.micro.magnet.fsu.edu/…/mitochondria.html)

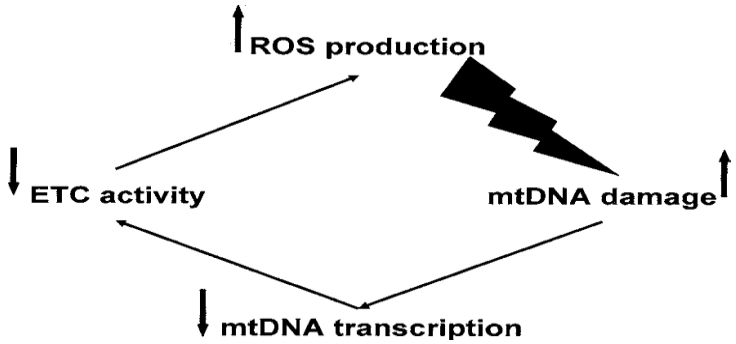


Mitochondria are self-replicating and contain their own double-stranded, circular DNA which, in *Homo sapiens*, is 16,569 bp in length (Alexeyev et al., 2004). A cell may contain only one mitochondrion, but hundreds, or even thousands of mitochondria are typically present in cells with substantial energy requirements. Mitochondria contain their own DNA (mtDNA) which is a compact genome encoding only 13 polypeptides of OXPHOS, along with rRNAs (ribosomal RNAs; 12S and 16S rRNA) and 22 tRNAs (transfer RNAs) required for the synthesis of these proteins.

The exact number of mammalian mitochondrial proteins is not currently known, but estimates based on comparisons to *Rickettsia prowazakeii* (Andersson et al., 1998), comparisons to *Saccharomyces cerevisiae* (Kumar et al., 2002), and two-dimensional gel electrophoresis studies of isolated mammalian mitochondria (Lopez et al., 2000; Rabilloud et al., 1998) suggest that the organelle contains approximately 1500 proteins. Only 13 proteins involved in oxidative phosphorylation are encoded by the mitochondrial genome. Most of the estimated 1500 human mitochondrial proteins involved in mitochondrial function are nuclear encoded, synthesized in the cytosol and targeted to mitochondria (Cotter et al., 2004). Although several recent studies have utilized proteomic and genetic approaches to expand the inventory of mammalian mitochondrial proteins, only

600–700 mitochondrial proteins are currently known (Da Cruz et al., 2003; Lopez et al., 2000; Ozawa et al., 2003; Taylor et al., 2003; Westermann and Neupert, 2003).

Figure 2: Diagram showing 'Vicious cycle' of mtDNA damage (Alexeyev et al., 2004)



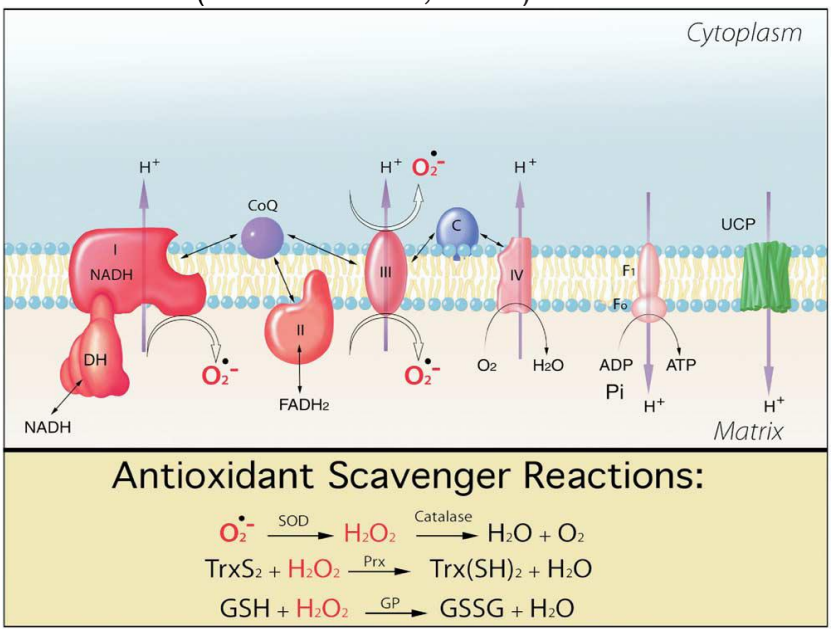
FUNCTIONS OF MITOCHONDRIA

Mitochondria are best known for housing the oxidative phosphorylation (OXPHOS) machinery as well as enzymes needed for free fatty acid metabolism and the Kreb's cycle. Key steps of heme biosynthesis, ketone body generation, and hormone synthesis also reside within this organelle (Stryer, 1988). The mitochondrion generates the majority of cellular reactive oxygen species (ROS) and has specialized scavenging systems to protect itself and the cell from these toxic by-products. The most prominent role of mitochondria is to produce ATP (i.e., phosphorylation of ADP) through respiration, and to regulate cellular metabolism (Voet et al., 2006). It is also play role in heat production (Nicholls et al., 1973) and storage of calcium ions (Lehninger et al., 1970). They are important participants in redox-dependent intracellular signaling (Droge, 2002).

The other important metabolic functions of mitochondria are the breakdown of the fatty acids by β-oxidation and breakdown of some amino acids. Mitochondria are involved in other cellular functions such as Ca^{2+} homeostasis, Fe/s clusters and apoptosis. Several lines of evidence indicate that mitochondria are involved in programmed cell death or apoptosis (Cortopossi and Wong, 1999; Green and Reed, 1999; Wallace, 1999). Through the opening of the inner membrane permeability

transition pores of mitochondria; the process of apoptosis by mitochondria is initiated (Green and Reed 1999; Wallace 1999; Figure 3).

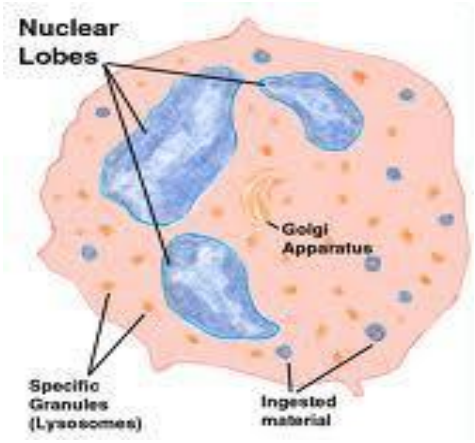
Figure 3: Diagram showing reactive oxygen species (ROS) production in Mitochondria (Balaban et al., 2005)



NEUTROPHILS

Neutrophil granulocytes are generally referred to as either Neutrophils or polymorphonuclear leucocytes (or PMNs). Neutrophils are the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. . Normally neutrophils contain a nucleus divided into 2-5 lobes. The average half-life of non-activated neutrophils in the circulation is about 12 hours. Neutrophils are the most abundant white blood cells in humans, they account for approximately 70% of all white blood cells (leukocytes). The stated normal range for human blood counts varies between laboratories, but a neutrophil count of 2.5-7.5 x 10^9/L is a standard normal range. Neutrophils protect the host against pyogenic (pus causing) microorganisms and participating in the inflammatory process which aids in walling off and destroying traumatized tissue (Raam et al., 2006). In addition to recruiting and activating other cells of the immune system, neutrophils play a key role in the front-line defence against invading pathogens. Neutrophils have three strategies for directly attacking micro-organisms: phagocytosis (ingestion), release of soluble anti-microbials (including granule proteins) and generation of neutrophils extracellular traps (NETs).

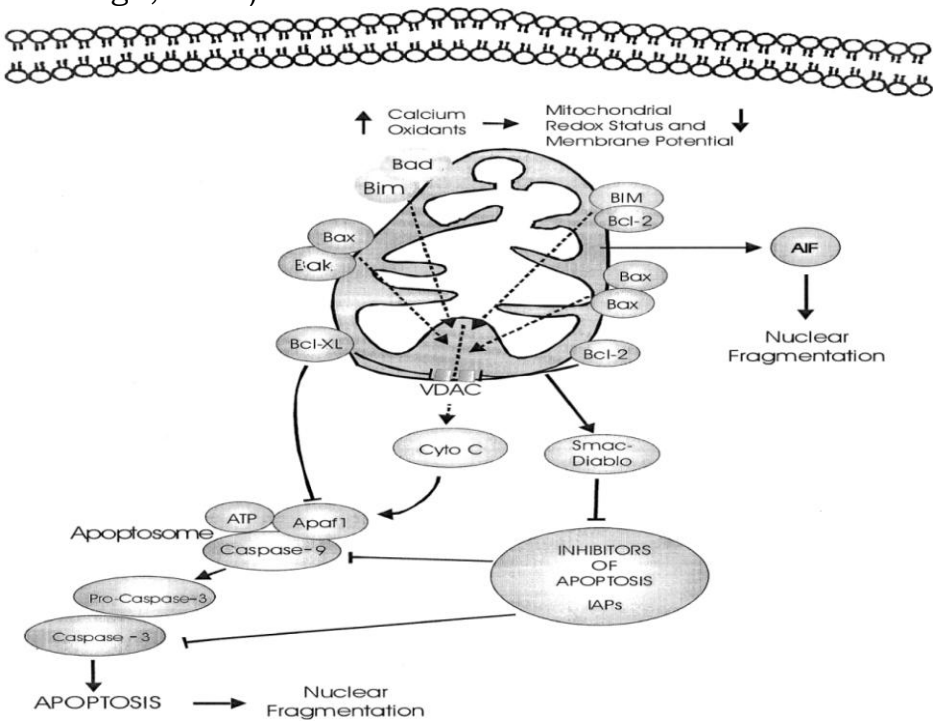
Figure 4: Diagram showing morphology of Neutrophil (www.education.vetnet.vt.edu/…/labs/lab6.html)



Role of Mitochondria in Neutrophils

It is commonly assumed that human neutrophils possess few, if any; functional mitochondria and they do not depend on these organelles for cell function (Fossati et al. 2003)**.** It has been shown that the mitochondria possess additional functions in cell death (Apoptosis). These organelles contain a number of proteins in the inter membrane space, which, once released into the cytosol, induce and/or amplify the activation of apoptotic caspases (Maianski et al., 2004). It is also find out that presence of mitochondrial network in Neutrophils participates in regulation of cell shape and chemotaxis (Fossati et al., 2003).

Figure 5: Diagram showing Mitochondrial mediated pathway of Apoptosis (Pollack and Leeuwenburgh, 2001)



NEUTROPHIL AND APOPTOSIS

According to Marinos et al., (1981), in humans the number of mitochondria present in immature and inactive somatic cell is 87±10 and in highly active state is 600±77. Central in the regulation of the short life span of neutrophils are their mitochondria. These organelles hardly contribute to the energy status of neutrophils but play a vital role in the apoptotic process. Not only do the mitochondria contain cytotoxic proteins that are released during apoptosis and contribute to caspase activation, but they also act as sensors of the metabolic and redox state of the cell and as scavengers of free Ca^{2+}. The balance of the expression and activity of the proapoptotic and antiapoptotic members of the Bcl-2 family of proteins determines the life span of neutrophils, because these proteins are essential for the formation of a permeability transition pore in the mitochondria and also seem to control the release of Ca^{2+} from the endoplasmic reticulum and thereby mitochondrial energy metabolism (Raam et al., 2006; Figure 5).

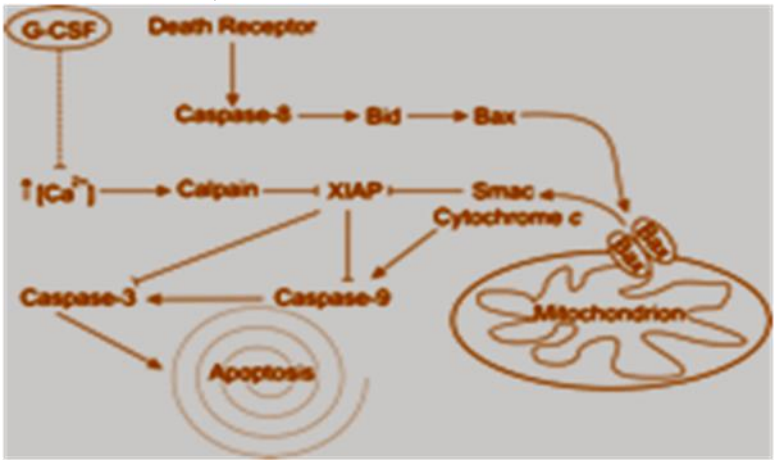
Neutrophil mitochondria hardly participate in ATP synthesis, and have a very low activity of the tested marker enzymes. Moreover, two important mitochondrial enzymes, glutamate dehydrogenase (GDH) and fumarase, which are often used as

markers of mitochondria, displayed a borderline low activity in neutrophils (Maianski et al., 2004). Mitochondria are a second, less prominent source of ROS in Neutrophils (Bassoe et al., 2003). Recent studies have shown that Neutrophil mitochondrial activity, although not essential for phagocytosis or respiratory burst initiation, does affect chemotaxis (Fossati et al., 2003) and apoptosis (Genestier et al., 2005; Scheel-Toellner et al., 2004).

Neutrophils, the primary phagocytic cells of the human immune system, have a very short life span after leaving the bone marrow of approximately 24 hours. Thereafter, they die either by intrinsically or extrinsically induced apoptosis. Extrinsically, neutrophil apoptosis can be activated through the ligation of death receptors, such as Fas/CD95 or other receptors of the tumor necrosis factor alpha (TNFα) family (Lies et al., 1996; Renshaw et al., 2003). Intrinsically, neutrophil apoptosis appears to be regulated at the level of their mitochondria and involves the spontaneous clustering of death receptors (Scheel-Toellner et al., 2004) or release of cathepsin D (Conus et al., 2008).

G-CSF (Granulocyte- Colony Stimulating Factor), inhibits neutrophil apoptosis by the inhibition of calpain activity. Calpains are a family of calcium-dependent cysteine proteases. Calpain-1 has been shown to be involved in the activation of the pro-apoptotic Bcl-2 family member Bax in human neutrophils (Altznauer et al., 2004). Results show that G-CSF inhibits the activation of calpains and limits the increase in intracellular Ca^{2+} during neutrophil apoptosis. As an apparent consequence of this inhibition, XIAP degradation is prevented and the downstream activation of caspase-9 is delayed, resulting in inhibition of cell death. Thus, G-CSF controls neutrophil apoptosis downstream of the mitochondria at the level of caspase activation (Raam et al., 2008; Figure 6).

Figure 6: Schematic representations of the apoptotic pathways in neutrophils (Raam et al., 2008)



In the study, Lizzia Raffaghello has addressed for the first time the modulation of neutrophil function by MSC (Mesenchymal Stem Cell). He demonstrates that human MSC potently inhibit in vitro apoptosis of both resting and activated neutrophils at very low MSC: neutrophil ratios (up to 1:500). Prolongation of neutrophil survival by MSC was found to involve reciprocal modulation of two mitochondrial proteins of the Bcl-2 family, Bax and MCL-1, but not of CD95. Expression of proapoptotic Bax increases in neutrophils undergoing spontaneous apoptosis, whereas that of antiapoptotic MCL-1 is upregulated in surviving neutrophils Accordingly, it was concluded that the protection of neutrophils from apoptosis provided by MSC was paralleled by Bax downregulation and MCL-1 upregulation in the former cells (Moulding et al., 1998; Dibbert et al., 1999).

Reactive oxygen species (ROS) production is an essential step required for the elimination of invading pathogens by neutrophils. This is exemplified by chronic granulomatous disease, a genetic disorder characterized by defective production of superoxide metabolites, in which neutrophils display impaired intracellular killing of ingested microorganisms (Dinauer and Orkin, 1992).

In 1982, Harvey Carp has found that Mitochondrial N-Formylmethionyl proteins act as chemo-attractants for neutrophils. A number of agents of both host and microbial origin posess potent PMN (Poly Morphonuclear Leukocytes) chemotactic activity which includes complement fragments, oxidised lipids, connective tissue

breakdown products as well as factors derived from macrophages and bacterial cells. All of these are agents are believed to play an important role in the accumulation of PMN at sites of inflammation in vivo. Schiffmann et al., (1975) proposed that the bacterial factors could consist of N-formylmethionine containing peptides derived from bacterial proteins. The mitochondrial protein synthetic apparatus uses N-Formylmethionine to initiate protein synthesis (Schatz and Mason, 1974; Mahler, 1979), in a manner similar to prokaryotes.

N-Formylmethionyl mitochondrial proteins could be chemotactic for PMN, in manner similar to bacterial and synthetic N-Formyl-methionine peptides. He reprted that disrupted human mitochondria stimulate PMN chemotaxis in vitro. He also found that nonformylated mitochondrial proteins, tested under the same conditions, were not chemotactic (Carp, 1982).

Study suggested that mature neutrophils express functional GPR109A (Nicotinic Acid receptor) receptors, which have been known to inhibit lipolysis in adipocytes (Tunaru et al., 2003). Receptor expression in the neutrophilic lineage appears to be induced during the terminal differentiation phase since a mixed population of immature bone marrow neutrophils did not demonstrate evidence for its expression. Moreover, GPR109A expression was not seen in mature eosinophils, suggesting that it is not a general feature of all granulocytes. Recently, it has been demonstrated that the receptor is also expressed on macrophages and dendritic cells, but not on monocytes (Benyo et al., 2005). Therefore, GPR109A receptors are expressed on some, but not all, leukocytes and may play a role in defense mechanisms against pathogens, at least within the innate immune system. Activation of GPR109A by Nicotinic Acid (NA) resulted in decreased levels of cyclic adenosine monophosphate (cAMP), most likely due to Gi-mediated inhibition of adenylyl cyclase activity.

NA-induced apoptosis was reversed by the addition of cell-permeable cAMP, pointing to the possibility that reduced cAMP levels promote apoptosis in neutrophils. Neutrophil apoptosis has previously been reported to be regulated by cAMP. For instance, agents and inflammatory stimuli that increase cAMP levels

delayed spontaneous neutrophil apoptosis (Rossi et al., 1995). PKA is a major target of cAMP (Zaccolo et al., 2005) and may therefore be involved in cAMP-mediated antiapoptosis in neutrophils. However, the targets of PKA responsible for this effect remain to be determined. PKA is known to phosphorylate multiple targets, including Bad, a known pro-apoptotic member of the Bcl-2 family (Affaitati et al., 2003). Phosphorylated Bad is considered as being antiapoptotic, since it is bound in the cytosol to 14-3-3 proteins (Raff, 1998). Therefore, hypothesized that NA-mediated decreased cAMP levels and subsequent decreased PKA activity may reduce Bad phosphorylation levels. Indeed, in the presence of NA, Bad demonstrated decreased phosphorylation at Ser 136. Moreover, they obtained evidence that less Bad is sequestered in the cytosol by 14-3-3 proteins as the consequence of NA treatment. Interestingly, among the different phosphorylation sites of Bad, Ser 136 appears dominant in 14-3-3 binding (Chiang et al., 2003). Data suggest that less phosphorylated Bad as a consequence of NA stimulation is indeed pro-apoptotic and largely contributes to accelerated neutrophil apoptosis.

MITOCHONDRIA, ANTIOXIDANTS AND AGING

It has been reported that cardiolipin is involved in age linked decline of Cytochrome C Oxidase activity in rat heart mitochondria (Paradies et al., 1993). According to Vitorica et al., (1981) there is partial impairment of Krebs cycle function, and a reduced energy-producing capacity in the aged rat heart. It has been reported that there is the increased level of lipid peroxidation and increased LDH activity in serum and neutrophil with smoking habit (Mahapatra et al., 2008). There is decrease activity of GSH-Px and SOD during ageing. Lipids peroxidation, expressed in term of MDA formation decreases in aged rats (Cand et al., 1989).

According to McElroy et al., (1992) the developmental expression of antioxidant enzymes differs between tissues and that, only increased expression of catalase activity is associated with human lung development. Phaneuf and Leeuwenburgh (2002) found that mitochondrial MnSOD and GPX activities were significantly elevated in the old animals, possibly suggesting that they are upregulated in response to increases in superoxide radicals and hydrogen peroxide.

Aging has recently been shown to promote lipid peroxidation of mitochondrial membranes by a mechanism involving chaotropic oxidants. Nohl (1979) reported the relationship between these membrane alterations and the activities of lipid-dependent enzymes of isolated heart mitochondria from 3 month and 24 month old rats. Analysis of the membrane lipids by gas-liquid chromatography reveals a distinct age-dependent fall in the content of polyunsaturated fatty acids accompanied by a slow increase in the degree of fatty acid saturation. It is concluded from the results that aging influences enzyme-protein-lipid interactions by radical-induced peroxidation of the surrounding membrane lipids.

The mitochondrial theory of aging also encompasses the mechanisms that may lead to cellular senescence in contractile tissues (Figueiredo et al., 2008). Altered levels of mitochondrial activity in aged muscle tissues have been well established and extensively reviewed (Lanza and Nair, 2010). The detrimental accumulation of mitochondrial DNA deletions and mutations on the genetic level and deficiencies in the mitochondrial electron transport chain on the biochemical level are clearly associated with muscle aging.

The pathological consequences of an age-related decline in mitochondrial function are the impairment of essential ATP dependent cellular processes (Trifunovic and Larsson, 2008) and amplified oxidative stress in senescent tissues due to the increased release of reactive oxygen species from the mitochondrial respiratory chain (Chakravarti and Chakravarti, 2007).

In general, senescent muscle tissues are exposed to an enhanced production of mitochondrial reactive oxygen species, increased mitochondrial apoptotic susceptibility, disturbed mitochondrial bioenergetic functions, and a reduced transcriptional drive for mitochondrial biogenesis (Friguet et al., 2008; Figueiredo et al., 2009). Although these functional impairments clearly occur in skeletal muscle mitochondria during aging, biochemical studies have also demonstrated considerable age-related changes in the abundance and post translational modifications of key mitochondrial enzymes.

MITOCHONDRIAL PROTEIN

Table 1: Proteomic identification of mitochondrial proteins during skeletal muscle aging (Lisa, Kathleen and Kay, 2011)

Proteomic study	Changes in mitochondrial marker proteins	Reference
Analysis of total extracts from aged human vastus lateralis muscle	General increase in aerobic markers, including mitochondrial enzymes such as ATP synthase, ubiquinol cytochrome c reductase, and oxoglutarate dehydrogenase during muscle aging	Gelfi et al., 2006.
Analysis of total extracts from rat gastrocnemius muscle	Increase in mitochondrial enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, ATP synthase, and malate dehydrogenase during muscle aging	Doran et al., 2008.
Analysis of total extracts from rat gastrocnemius muscle	Differential effect on the abundance of mitochondrial isoforms of aconitase during muscle aging	O'Connell et al., 2007.
Analysis of total extracts from aged rat gastrocnemius muscle	Moderate effect on cytochrome c oxidase and isocitrate dehydrogenase during muscle aging	Piec et al., 2005.
Analysis of total extracts from rat gastrocnemius muscle	Increase in many enzymes involved in oxidative metabolism, such as ATP synthase, isocitrate dehydrogenase, ubiquinol-cytochrome c reductase, and pyruvate dehydrogenase during muscle aging	Capitanio et al., 2009.
Subproteomic study of the effect of aging and caloric restriction on rat muscle mitochondria	Increased levels of isocitrate dehydrogenase and malate dehydrogenase in aged muscle mitochondria. Caloric restriction appears to have only a marginal effect on the mitochondrial proteome	Chang et al., 2008.
Subproteomic analysis of mitochondria-enriched fraction from aged rat gastrocnemius muscle	Increased levels of mitochondrial creatine kinase, NADH dehydrogenase, ATP synthase, succinate dehydrogenase, and ubiquinol cytochrome c reductase during muscle aging	O'Connell and Ohlendieck, 2009.
Analysis of total extracts and mitochondria-enriched fraction from aged ratgastrocnemius muscle	Differential effect on mitochondrial enzymes, such as pyruvate dehyrdogenase, cytochrome c oxidase, isocitrate dehydrogenase, and ATP synthase during muscle aging	Lombardi et al., 2009.

Subproteomic analysis of mitochondria-enriched fraction from aged mouse hind limb muscles	Differential effects on the abundance and carbonylation of various mitochondrial enzymes, including NADH dehydrogenase, cytochrome c oxidase, and ATP synthase during muscle aging	Alves et al., 2010.
Analysis of detergent phase-extracted protein complement from aged rat gastrocnemius muscle	Increase in mitochondrial marker enzymes, such as ATP synthase and isocitrate dehydrogenase during muscle aging	Donoghue et al., 2010.
Proteomic analysis of nitration in aged rat skeletal muscle	Increased nitration levels in succinate dehydrogenase	Kanski et al., 2005.
Phosphoproteomic analysis of total extracts from aged rat gastrocnemius muscle	Decreased phosphorylation levels in cytochrome c oxidase and aconitase during muscle aging	Gannon et al., 2008.
Proteomic analysis of carbonylation in aged rat skeletal muscle mitochondria	Altered carbonylation levels in numerous mitochondrial proteins, including ATP synthase, NADH dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase during muscle aging	Feng et al., 2008.

Benard et al. (2006) investigated the physiological diversity in the regulation and control of mitochondrial oxidative phosphorylation. According to their study there was an important variability of mitochondrial morphology between tissues and observed a strong heterogeneity of mitochondrial intracellular distribution within tissues. The analysis of the relative expression level of seven proteins of the respiratory chain by Western blot indicates a higher content of complex I, II, III, IV, and V in heart- and muscle-isolated mitochondria than in liver, kidney and brain.

OBJECTIVES OF THE PRESENT INVESTIGATION

- To determine the Polymorphism at biochemical level.
- To perform gel electrophoresis for Mitochondrial Protein profiling.

MATERIALS AND METHODS

STUDY SUBJECT

Normal human male blood samples of reproductive age between 20-55 yrs were taken. These were grouped in following Age slab, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50 and 51-55. All the samples were collected from Sun Pathology Laboratory, Ahmedabad. This project has been approved by Institutional Ethical Committee (IEC/NU/I/IS/04).

PLAN OF WORK

The investigation has been divided into three stages. The Stage I included the collection of blood from the volunteers while the Stage II was subdivided into two stages i.e., blood was separated into blood cells and serum and hence stage II (a) was for the separation of serum and serum profiling and stage II (b) was for the isolation of neutrophil. Stage III was further isolation of mitochondria and its biochemical analysis from the neutrophils.

SAMPLE COLLECTION

Blood samples were collected for two purposes, one without anticoagulant for Serum biochemistry and anticoagulant containing blood for Neutrophil isolation and biochemical assays.

BLOOD PROFILING

Haematology of the blood samples were carried out in order to detect the variation in the hemoglobin (Hb), Total red blood corpuscles (RBC) and total white blood corpuscles (WBC) counts. Packed Cell Volume (PCV), Mean Corpuscular volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) recorded according to Natelson (1951) and Lynch et al., (1969).

Blood profiling is used to determine the health status of an individual. Mean corpuscular volume (MCV) is a measurement of the average size of your RBCs. The MCV is elevated when RBCs are larger than normal (macrocytic), for example in anemia caused by vitamin B12 deficiency. When the MCV is decreased, RBCs are

smaller than normal (microcytic) as is seen in iron deficiency anemia or thalassemias.

Mean corpuscular hemoglobin (MCH) is a calculation of the average amount of oxygen-carrying hemoglobin inside a red blood cell. Macrocytic RBCs are large so tend to have a higher MCH, while microcytic red cells would have a lower value.

Mean corpuscular hemoglobin concentration (MCHC) is a calculation of the average concentration of hemoglobin inside a red cell. Decreased MCHC values (hypochromia) are seen in conditions where the hemoglobin is abnormally diluted inside the red cells, such as in iron deficiency anemia and in thalassemia. Increased MCHC values (hyperchromia) are seen in conditions where the hemoglobin is abnormally concentrated inside the red cells, such as in burn patients and hereditary spherocytosis, a relatively rare congenital disorder.

SERUM ISOLATION AND SERUM BIOCHEMISTRY

Serum biochemistry refers to the chemical analysis of serum, which is a major component of blood. The analysis can include many different tests, each of which provides information about one or more organs in the body. If a test result is abnormal, it may indicate that disease is present. Further assessment of the test results may indicate which organ system is affected, and may provide information about the nature and severity of the problem.

Non heparinised blood is centrifuged at 3000 rpm for 10 minute at room temperature. Serum samples were taken for biochemical assay using reagent kits (Accucare Ltd. Mumbai, India). Different biochemical assays were performed which includes, Total Protein assay, Cholesterol, Triglyceride assay, CK-MB assay, Urea, LDH, SGOT (Serum glutamate oxalate transaminase), SGPT (Serum glutamate pyruvate transaminase).

The two main types of protein found in blood are called Albumin and Globulin. These proteins can be measured individually, or combined in a single test called Total Protein. Albumin levels can indicate if a patient is dehydrated, and

can provide information about the function of the liver, kidneys and digestive system. Globulin levels reflect underlying inflammation or antibody production. Increased levels of globulins are often associated with infectious diseases, immune-mediated disease, and some types of cancer.

The most commonly used liver tests measure Alanine aminotransferase (ALT), Aspartate aminotransferase (AST). These two enzymes are often increased when there is liver cell inflammation, injury, or destruction. The two substances most commonly measured to assess kidney function are Urea (also called blood urea nitrogen or *BUN*) and Creatinine. Urea is a by-product of protein breakdown; it is produced in the liver and excreted from the body in urine. Increases in BUN may indicate dehydration, gastrointestinal bleeding, or kidney disease. Decreases in BUN are associated with overhydration, liver failure, or kidney disease. Creatinine is a by-product of muscle metabolism. Increased levels of creatinine indicate decreased kidney function.

Cholesterol is produced in the liver as part of fat metabolism. Increases in cholesterol are associated with hormonal and metabolic diseases, liver disease, and serious kidney disease (www.fetchdog.com/…/Serum-Biochemistry-Profile/…/AR000010045).

Serum testosterone assay was performed by Chemiluminescence method at Scientific Diagnostic centre, Ahmedabad.

NEUTROPHIL ISOLATION

EDTA containing anticoagulated blood samples were taken for Neutrophil isolation (Kalmar et al., 1988). According to this protocol, Mono-Poly Resolving Medium (M-PRM) (MP Biomedicals) is used for the efficient separation of blood cells. M-PRM is a solution composed of a polysaccharide (Ficoll 400) and a radiopaque contrast medium (Hypaque) in a specific ratio to yield a density of 1.114±0.002. This unique mixture enables the resolution of both mononuclear and polymorphonuclear leucocytes into two distinct bands.

Isolated Neutrophil samples were taken for ultrasonication. Neutrophils were suspended in PBS having pH of 7.4. This suspension was sonicated four times at 50% setting, for 15 seconds each with 15 second rest between each sonication (Mclean et al., 1993).

ANTIOXIDANT ASSAY

The following defensive enzymes related with oxidative stress were assayed according to the reported methods. For this assay, sonicated cell suspension was used.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was measured as per protocol adopted from Kono, 1978. In the assay, superoxide ions (O_2-), generated by xanthine oxidase (XOD) conversion of xanthine to uric acid and hydrogen peroxide converts NBT to NBT-diformazan, which absorbs light at 540 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity present in an experimental sample. The activity of SOD was measured by monitoring the rate of inhibition of NBT (Nitro blue tetrazolium) reduction at 540 nm. One unit is defined as the amount of enzyme, which caused half-maximal inhibition of NBT reduction.

Catalase

Catalase activity was measured as per protocol adopted from Aebi, 1984. Decrease in absorbance due to the disappearance of H_2O_2 was recorded by spectrophotometer at an interval of 1 minute up to 5 minutes at 514 nm at 25°C.

MITOCHONDRIAL ENZYMES

In the present investigation, NAD^+ malate dehydrogenase was used as marker for the inter membrane matrix activity and functionality and Succinate dehydrogenase was used for the inner membrane of the mitochondria.

Malate Dehydrogenase

Activity was measured as per the protocol adopted from Ochoa (1955). Malate dehydrogenase catalyses the reversible, pyridine nucleotide dependent dehydrogenation of L-malate to oxaloacetate.

L-Malate + NAD^+ $\rightleftharpoons$ Oxaloacetate + NADH + H^+

The reaction is followed spectrophotometrically by measuring the rate of NADH oxidation at 340 nm in the presence of enzyme and oxaloacetate. One unit is the amount of enzyme catalysing the oxidation of 1.0µmole of NADH per minute.

Succinate Dehydrogenase

Succinate Dehydrogenase is marker enzyme of Mitochondria. Succinate Dehydrogenase catalyses the oxidation of succinate to fumarate. This reation is measured by monitoring the reduction of an artificial electron acceptor. To use an artificial electron acceptor, the normal path of electrons through the mitochondrial electron transport system must be blocked. This is accomplished by adding either sodium azide or potassium cyanide. These poisons inhibit the transfer of electrons from Cytochrome a_3 to the final electron acceptor, oxygen thus electrons cannot be passed along by the preceding cytochromes and coenzyme Q. Instead, the electrons from SDH-$FADH_2$ can be picked up by an artificial electron acceptor, such as the dye 2,6dichlorophenolindophenol. The reduction of DCIP can be followed spectrophotometrically, since the oxidized form of the dye is blue and the reduced form is colourless. The change in absorbance, measured at 600 nm (Spencer and Guest, 1973).

SDH-$FADH_2$ + DCIP oxi. $\longrightarrow$ SDH-FAD + DCIP red. + $2H^+$

Blue Colourless

MITOCHONDRIAL PROTEIN PROFILING

The sonicated neutrophils were then subjected to protein profiling by SDS-PAGE. Protein samples were prepared in Tris HCL (1M, pH 6.8), 20% SDS, glycerol, β-mercapto and bromophenol blue (tracking dye). Samples were kept in a boiling water bath for 5 min and then on ice for the same time. Processed samples were loaded into the wells in the stacking gel. The electrophoresis unit is connected to a power supply which is run at constant 50 V, then increased to 150 V just after all the dye moved into the running gel.

STATISTICAL ANALYSIS

Data were expressed as median ± S.E. The statistical significance was evaluated by one way analysis of variance (ANOVA) using Statfi software.

RESULTS AND DISCUSSION

BLOOD SAMPLES

In the present investigation, all the blood samples were collected from Sun Pathology Laboratory, Ahmedabad. In all ninety blood samples were obtained. They groups into various age groups, viz, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50 and 51-55 years. The numbers of samples obtained in each age group are as follows 16, 14, 9, 14, 6, 13 and 18 respectively.

BLOOD PROFILING

There was no significant change observed in any of the parameters of blood profiling, viz., Hb, RBC, WBC and platelets. There was slight increase in Hb values of 41-50 age groups which and decreased in the subsequent age group i.e., 51-55. Similar trend was also observed in the total RBC count. Hematocrit values of the various age groups did not show much changes with respect to increasing age but the values fluctuated among the age groups (Table 2 a; b).

All the values are expressed in Mean±SD. The number of samples taken are *-16, **-14, @-9, $-14, ^-6, ■-13 and ▲- 18

Table 2 (a): Blood Profiling of different age groups in male.

Age group	Hemoglobin (gm %)	RBC (10^6/c.mm)	WBC (10^3/c.mm)	Platelets (10^5/c.mm)
21-25*	15.1±1.15	5.4±0.57	7.4±0.87	2.7±0.31
26-30**	14.3±0.07	4.8±0.13	8.4±5.11	2.6±0.15
31-35@	14.5±0.90	5.0±0.31	11.5±7.94	3.0±0.86
36-40$	14.3±1.25	5.0±0.21	9.8±3.83	3.0±0.43
41-45^	14.7±0.21	5.4±0.49	6.9±0.11	2.8±0.37
46-50▪	14.9±1.97	5.2±0.59	6.6±1.05	3.0±0.92
51-55▲	14.5±1.56	5.1±0.74	7.7±1.99	2.9±0.32

Table 2(b): Hematocrit values of different age groups

Age group	PCV (%)	MCV (fL)	MCH (pg)	MCHC (%)
21-25*	44.3±2.00	81.4±4.02	28.2±1.04	34.7±1.15
26-30**	41.0±2.05	84.5±1.83	29.4±0.98	34.9±1.09
31-35@	41.6±2.01	83.6±5.16	29.1±2.57	34.8±0.97
36-40$	41.6±2.98	83.6±2.99	28.8±1.92	34.4±1.56
41-45^	43.3±0.63	80.3±6.15	27.2±2.82	33.9±0.98
46-50▪	42.7± 4.57	80.9±4.13	28.2±0.99	34.2±2.01
51-55▲	42.2±3.33	83.2±8.84	28.6±3.19	34.4±1.35

SERUM BIOCHEMISTRY:

There was no demarcated significant change in the values of all the serum biochemical parameters. All the levels fluctuated from 21-25 age group to 41-45 age group. Subsequently there was a declining trend observed in all the parameters (Table 3 a; b).

Table 3(a): Serum Biochemistry of different age groups

AGE GROUP	TOTAL PROTEIN (g/dL)	TRIGLYCERIDE (mg/dL)	CHOLESTEROL (mg/dL)	UREA (mg/dL)
21-25*	6.7±0.52	104.8±26.82	120.9±45.47	42.4±19.77
26-30**	7.4±0.81	92.8±38.78	148.9±31.64	23.3±3.78
31-35@	7.1±1.67	132.1±77.83	159.0±23.39	35.9±5.07
36-40$	6.8±0.87	97.8±45.83	172.5±61.44	28.6±14.64
41-45^	6.9±1.09	145.7±77.07	200.0±10.37	36.1±8.06
46-50▪	7.3±0.66	130.2±58.60	190.0±10.15	39.1±21.95
51-55▲	7.1±1.00	73.6±25.05	184.2±64.16	34.2±7.97

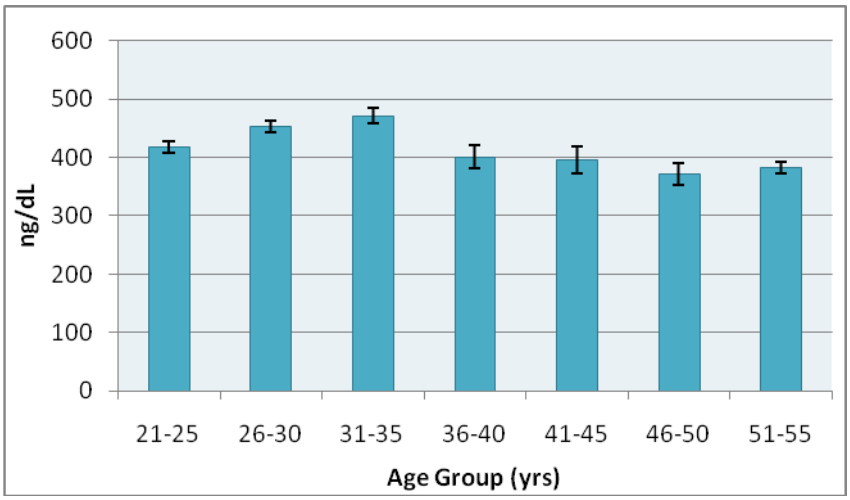
Table 3(b): Serum Biochemistry of different age groups

AGE GROUP	LDH (U/L)	SGPT (U/I)	SGOT (U/I)	CK-MB (U/I)
21-25*	105.2±8.65	23.8±2.15	41.7±3.71	21.6±2.46
26-30**	98.9±9.32	19.1±2.25	39.4±5.22	17.3±1.94
31-35@	73.3±8.16	8.7±6.98	37.2±26.42	15.9±5.25
36-40$	82.9±32.63	14.5±1.20	39.3±45.69	19.4±2.75
41-45^	80.7±5.46	10.5±0.00	25.2±1.75	21.5±3.65
46-50▪	76.2±2.59	13.0±0.88	33.7±0.00	17.4±1.42
51-55▲	66.8±8.51	13.0±5.47	43.0±9.81	15.4±4.34

Serum Testosterone

Serum testosterone levels show gradual increasing level till the age group 31-35. In the 31-35 age group it shows highest amount after that the levels are decreasing with age.

Graph 1: Serum testosterone levels in different age group



NEUTROPHIL ISOLATION

Using M-PRM medium, neutrophils were isolated.

FIGURE 7: Showing Neutrophil isolation.

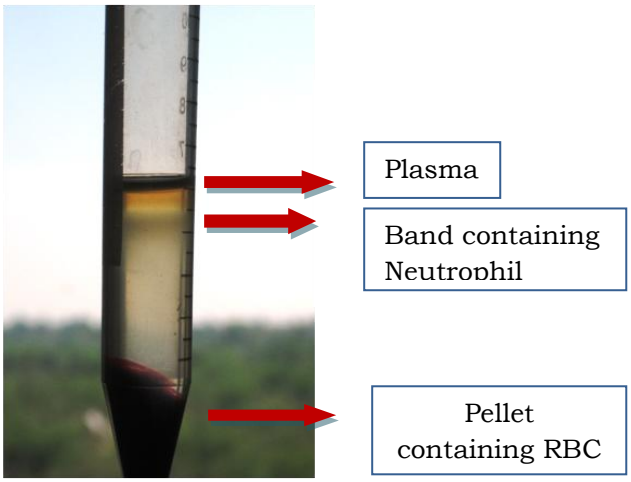
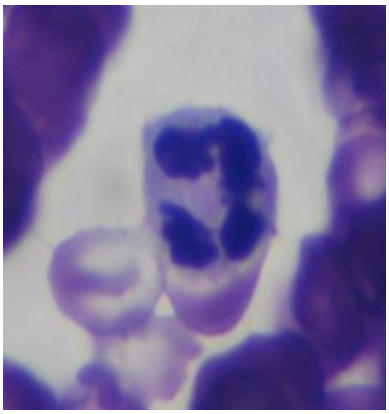


FIGURE 8: Showing Multilobed Neutrophil (centre).

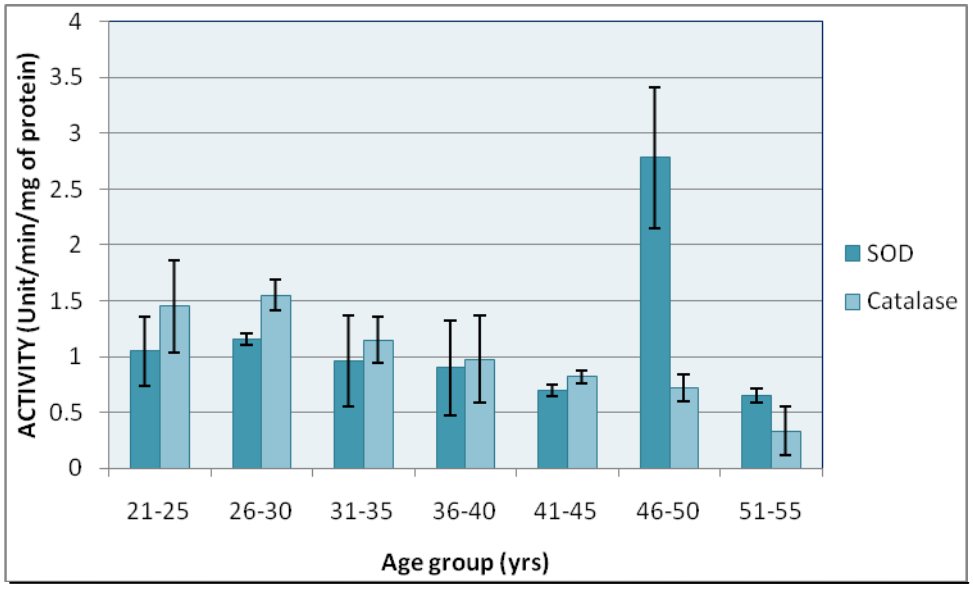


ANTIOXIDANT ASSAYS

Superoxide dismutase (SOD) and Catalase mainly act as antioxidant enzymes. They act against Reactive Oxygen Species (ROS) and protect cell against free radical damage.

The SOD and Catalase levels showed a gradual decrease with increasing age. In 46-50 age group only the SOD levels significant increase which again decreased drastically in the subsequent age group (Graph 2).

Graph 2: Comparison of SOD and Catalase activity in different age group



Malate Dehydrogenase (MDH)

The level of MDH was seen in neutrophil. Data is showing that MDH activity is fluctuating in different age group. There is increased value is seen in the 36-40 and 51-55 age group (Table 4; Graph 3).

Table 4: Activity of MDH in neutrophil in different age group

Age Group	Malate Dehydrogenase (Units/mg of protein)
21-25*	2.42±1.2
26-30**	1.55±0.24
31-35@	2.28±0.98
36-40$	4.29±2.29
41-45^	1.81±0.19
46-50▪	1.45±0.7
51-55▲	3.09±0.95

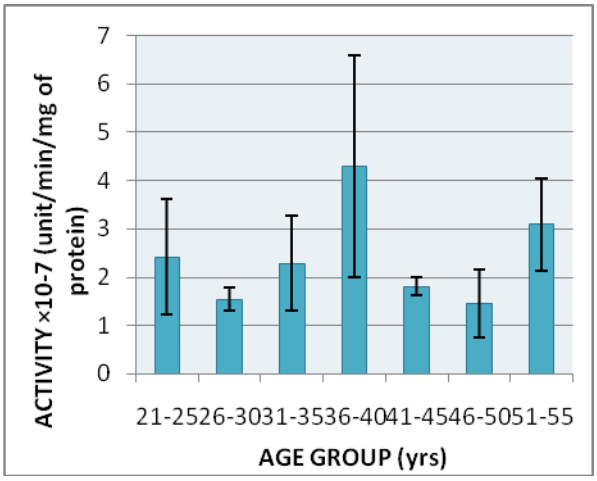
Succinate Dehydrogenase (SDH)

Succinate Dehydrogenase is known as mitochondrial marker enzyme. Here the activity of this enzyme in neutrophil is showing variation in different age group. Here activity is increased in the age slab of 36-40 and 41-45. Then again activity decreases (Table 5; Graph 4).

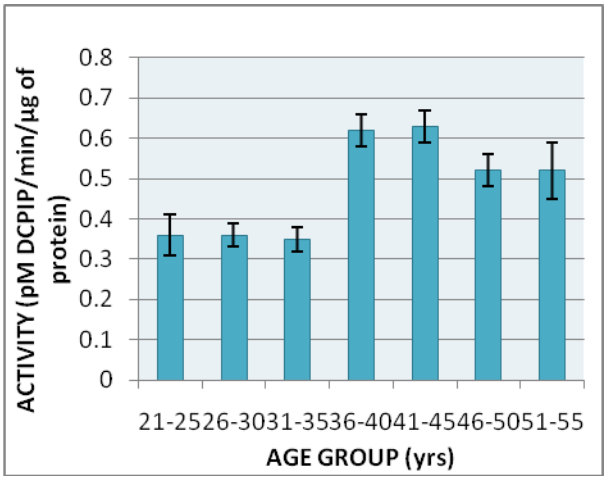
TABLE 5: Activity of SDH in Neutrophil in different age group

Age group	Succinate Dehydrogenase (pM DCIP/min/µg of protein)
21-25*	0.36±0.05
26-30**	0.36±0.03
31-35@	0.35±0.03
36-40$	0.62±0.04
41-45^	0.63±0.04
46-50▪	0.52±0.04
51-55▲	0.52±0.07

Graph 3: MDH Activity in Neutrophil



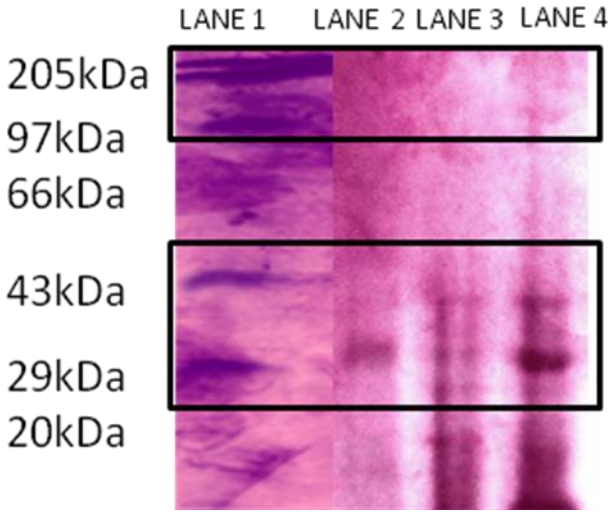
Graph 4: SDH activity in neutrophil



MITOCHONDRIAL PROTEIN PROFILING

In the present investigation, the band of 29 kDa Molecular weight (Mol. Wt) was found in all the age group that is, in 21-25, 26-30 and 51-55. In the case of 51-55 age group band of 29 kDa Mol. wt was found most prominent. In the age group of 26-30 and 51-55 band of approximately 30 kDa was also found. In the case of high molecular protein band, was only found in 51-55 age groups but not clearly seen in other age group. Band of 43 kDa is also observed in the last two age groups. High mol. wt band is observed in 51-55 age group but not clearly seen.

Figure 9: Mitochondrial protein profiling of neutrophil of 21-25, 26-30 and 51-55 age group



Lane 1: Ladder; Lane 2: 21-25 age group; Lane 3: 26-30 age group and Lane 4: 51-55 age group.

DISCUSSION

Haematology is carried out to check the health status of an individual. It has been found that the reference range varies, depending on the age, sex and race of a population, and even the instruments that are used in laboratory to perform the tests. Here, in the present study, the values of all blood profiling parameters estimated, did not show any trend with increase in the age. Data revealed that the values are fluctuating within the same age group as well as among the other age groups. This variation could be attributed due to dietary factor, environmental factor or due to any addiction. In the present study, one of the factors for the variation could be number of samples also. As the numbers of samples were less it might have resulted in the fluctuations within the various age groups.

Serum biochemistry includes many tests and each of which provides information about one or more organs of the body. If increased values of serum biochemistry are observed than the normal values, then it can be directly co-related with abnormal functioning of the related organs. Here, serum biochemical levels with increasing age are shown (Table 3 and Figure 8). Data showing variations in the values of serum biochemical levels in different age slab. All the values fluctuated within the normal values. Variations observed, can be attributed due to various factors like, environment, diet, health, addictions, pre-medical history and drug usage of the individuals.

Aging has been associated with a change in the levels of testosterone levels. In healthy, aging groups of men testosterone levels decline with age on the order of 100 ng/dl per decade (Morley et al., 1997). These hormonal fluctuations begin around the age of mid 40s and are well established by the age of 50 years as the individual enters into a phase of andropause.

However, there is great inter-individual variability in testosterone levels among healthy older men has been noted. Testicular failure, due to a decline in Leydig cell mass, is thought not to be the only reason for the hormonal changes described.

The increase in LH (luteinizing hormone) levels, for example, is relatively small, except for men older than 70 years, and should be higher in middle- aged and "young old" men if a primary testosterone deficiency was the problem (Vermeulen, 1993). It is believed that the relatively small increase in LH levels, along with a decrease in relative responsiveness of LH and FSH (Follicle-stimulating hormone) to GnRH (Gonadotropin-releasing Hormone), in elderly men is more consistent with a failure of the hypothalamic-pituitary system and that the decline in testosterone levels (Sternbach, 1998).

In the present study the levels of serum testosterone (Graph 1) shows gradual increased levels till the age of 35 yrs after that decreased value was observed with increasing age.

ROS are usually derived from abnormally interrupted metabolism of oxygen and thought to play an important role in oxidative damage to biological macromolecules. The mitochondrial electron transport chain is widely viewed as the main site in the cell for Superoxide radical and H_2O_2 generation. The reduction of molecular oxygen in the cells involved the cytochrome *a/a*3 complex or Cytochrome oxidase, which catalyzes the transfer of four electrons from reduced cytochrome *c* to molecular oxygen. Although molecular oxygen is a good electron acceptor, a partial or incomplete transfer provides a flux of oxygen radical from mitochondria, subsequently leading to an excessive formation of ROS in the biological system. Increasing evidence indicates that oxygen radical production in the cell increases with age in mammals and insects. The age-related increase in production of pro oxidants may be derived from the membrane damage by super oxide radical and H_2O_2. This may lead to accumulation of oxidatively damaged macromolecules, including DNA, RNA, lipids, and crucial enzyme proteins in senescent cells (Tian et al., 1988)

Antioxidant enzymes are considered to be a primary defence that prevents biological macromolecules from oxidative damage. SODs rapidly convert superoxide radical to less dangerous H_2O_2, which is further degraded by catalase (CAT) to water. Thus, the steady-state level of antioxidant enzymes during aging

may protect some important tissues against free radical-mediated damage. In the present study, the SOD levels showed gradual decrease only 46-50 age group showed the significant increase in the activity which again decreased drastically in the subsequent age group.

CAT activity consistently decreases in mitochondria as a function of age (Table 4 and Figure 11b). CAT catalyzes the decomposition of H_2O_2 to produce water and molecular oxygen, and plays a major role in protecting cells against oxidative damage.

Similarly, over expression of superoxide dismutase and catalase significantly prolongs the life span of drosophila (Orr et al., 1994). It is, however, important to point out that this effect could not be reproduced in longer-lived strains of the fly (Orr et al., 2003). Further, supporting evidence comes from long-lived AGE-1 mutants of the nematode worm *Caenorhabditis elegans* Life span extension in these worms is accompanied by higher than normal expression of SOD and catalase (Larsen, 1993; Vanfleteren and Vreese, 1996). Although it seems that enhancing the antioxidant buffering capacity extends life span in several species, the reverse is not always true (i.e., decreasing ROS scavenging systems does not shorten life span).

As SOD and Catalase are of great significance and constitute the antioxidant system in mitochondria along with the other enzymes. With increasing age Catalase declines which can lead to an increase in ROS production and its accumulation in the cell. Eventually, this will lead to decrease cell activity, which may further result in apoptosis and cell death.

Mitochondria contain the biochemical machinery for the aerobic cellular respiration, the process by which sugars, fatty acids and amino acids are broken down to carbon dioxide and water, with some of their chemical energy captured as ATP. A key series of reactions in cell respiration is the krebs cycle, a complex pathway involving some nine enzymes and numerous metabolic intermediates. Succinate dehydrogenase is the unique among the Krebs cycle enzymes in that it

is tightly bound to the inner mitochondrial membrane. Malate dehydrogenase is found in mitochondrial matrix.

Considerable tissue-specific differences exist within the mitochondrial proteome and reflect the diversity of mitochondrial functions in individual organs (Mootha et al., 2003; Forner et al., 2006). In analogy, elevated levels of mitochondrial enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, ATP synthase, malate dehydrogenase, ubiquinol-cytochrome c reductase, and pyruvate dehydrogenase, were also shown to occur during the aging of rat gastrocnemius muscle (O'Connell et al., 2008; Capitanio et al., 2009). Chang and co-authors have studied the effect of aging and caloric restriction on the rat mitochondrial proteome. In skeletal muscles, isocitrate dehydrogenase and malate dehydrogenase were shown to be increased in 25- month-old Fisher 344 rats, as compared to 6-month-old rats. Caloric restriction appears to have only a minor effect on age-related changes in the mitochondrial protein complement (Chang et al., 2007).

Proteomic studies of posttranslational changes in aged skeletal muscle have revealed increased nitration levels in succinate dehydrogenase (Kanski et al., 2003; Gannon et al., 2008) and altered carbonylation levels in ATP synthase, NADH dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase during muscle aging (Schoneich, 2006).

In the present study, the levels of mitochoncrial enzymes showed a decline in activity with increase in age. In the mitochondria of neutrophil the activity of Succinate dehydrogenase and Malate dehydreogenase activity were showing fluctuations with increasing age. MDH activity increased in 36-40 and 51-55 age groups (Table 4; 5).

The SDS-PAGE results of the mitochondrial proteins did not reveal much information about the diversity and activity of the proteins with respect to the increase in age. The needs to be ascertained and further identification of the same needs to be done by 2D electrophoresis to confirm the types of enzymes and proteins affected with increase in the age of the individual.

CONCLUSION

From the present study, it can be concluded that, in normal men, there was no significant trend found in the blood profiling and serum biochemistry but, the serum testosterone showed decreased values with age from 36-40 group onwards. All the values are varying within the normal range. This work can be done again with more number of samples to check the variations in various age groups as less number of samples is one of the factors for these fluctuations.

The antioxidant enzymes SOD and Catalase in neutrophil mitochondria are showing decline in the activity with increasing age. It can be concluded that SOD and catalase can be used as one of the marker antioxidants for aging in neutrophil mitochondria.

The other mitochondrial enzymes such as Succinate dehydrogenase and Malate dehydrogenase are also showing decrease in their activity with increasing age. The polymorphism studies of these enzymes along with other apoptotic markers needs to be further studied to co-relate with mitochondrial functioning with age as a parameter.

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APPENDICES

APPENDIX I

Neutrophil isolation:

Procedure:

1. Place 3 ml of M-PRM into a sterile 13 x 100 mm test tube.
2. Carefully layer 3.5 ml of fresh, anticoagulant treated, human venous blood onto the medium. Blood must be used within 6 hours of collection.
3. Centrifuge at 300 x g for 30 minutes in a swinging bucket rotor at room temperature (15-30oC). Better separation is achieved by centrifuging at 2000 rpm (in a Beckman clinical centrifuge) for 45 min.
4. With a Pasteur pipette, draw off the plasma, take polymorphonuclear cell containing band in different tube.
5. Wash the cells with a balanced salt solution or culture medium. Cells should not remain in contact with the separation medium for extended periods of time as this could affect the viability of the cells.
6. Centrifuge at 250 x g for 10 minutes at room temperature.
7. Remove the supernatant.
8. Resuspend the cells in the culture medium appropriate to the application

Super Oxide Dismutase (Kono et al., 1978)

The enzyme SOD, catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. As such, it is important antioxidant defense in nearly cell exposed to oxygen. In humans, three isoforms of SOD are present. SOD1 is located in the cytoplasm, SOD2 in mitochondria and SOD3 in the extracellular region. The first is a dimmer, while the others are tetramers. SOD1 and SOD3 contain copper and Zinc, while SOD2 has manganese in its reactive centre.

Reagents:

1. 50 mM sodium carbonate buffer, pH 10.0.
2. 96 µM NBT in 67% DMSO (Dimethyl sulpoxide), solution should be freshly prepared.

3. 0.6% Triton X-100. 4) 20mM Hydroylamine hydrochloride, pH 6.0

Procedure

In the test cuvette, the reaction mixture contained the following: 1.3 ml sodium carbonate buffer (50 mM), pH 10.0, 500 µl NBT (96 µM) and 100 µl triton X-100 (0.6%). The reaction will be initiated by addition of 100 µl of hydroxylamine hydrochloride (20 mM), pH 6.0. After 2 min, 50 µl enzyme sample will be added and the percentage inhibition in the rate of NBT reduction will be recorded. One unit of enzyme activity will be expressed as inverse of the amount of mg protein required to inhibit the reduction of NBT by 50%

Calculation

SOD (unit/min/mg of protein): $\frac{\Delta A/30 \text{ sec} \times 4 \times 2}{\text{mg of protein}}$

Catalase (Aebi, 1984)

Catalase is common enzyme found in nearly all living organism. Its function includes catalyzing the decomposition of hydrogen peroxide to water and oxygen. Catalase has the highest turnover rate of all the enzymes. Catalase is tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin rings that allow the enzyme to react with hydrogen peroxide. The optimum pH for catalase is approximately 7. Its optimum temperature is 37^0C, which is approximately the temperature of the human body.

Reagent:

1. 0.2 M phosphate buffer, pH 7.0 containing 12 mM H_2O_2, freashly prepared.

Procedure:

The reaction mixtures (1 ml) contain 0.8 ml phosphate buffer (0.2 M, pH 7.0) containing 12mM H_2O_2 as substrate, 100 µl enzyme sample and distilled water to make up the volume. The decrease in absorbance/minute at 240 nm will be recorded against H_2O_2-phosphate buffer as blank.

Calculation:

Catalase (H$_2$O$_2$ degraded/min/mg of protein) $= \frac{\Delta A / 15 \text{ sec} \times 43.6 \times 4}{\text{mg of protein}}$

Malate Dehydrogenase (Ocoha, 1955)

Reagents:

1. 250 mM Phosphate buffer, pH 7.4
2. 1.5 mM NADH
3. 7.5 mM Oxaloacetate, pH 7.4
4. Enzyme solution to be assayed.

Procedure:

The reaction mixture contains 0.1 mL of buffer, 0.05 mL each of NADH, Oxaloacetate, enzyme and water to a final volume of 1.0 mL. The reaction is started by the addition of oxaloacetate and readings at 340 nm are made against a blank containing all components except NADH. The decrease in optical density between 30 and 34 seconds after the start of the reaction is linear and is used to calculate the enzyme activity. One unit is the amount of enzyme catalysing the oxidation of 1.0 µmole of NADH per minute.

Calculation:

Malate dehydrogenase (NADH oxidised/sec) $= \frac{\Delta A_{340} / 5 \text{ sec}}{6.2 \times 10^3}$

Succinate dehydrogenase (Spencer and Guest, 1973)

Succinate dehydroganase catalyses the oxidation of succinate to fumarate. In this rection, two hydrogen atoms are removed from Succinate by flavin adenine dinucleotide (FAD), a prosthetic group that is tightly attached to Succinate dehydrogenase.

Reagents:

1. Mannitol assay buffer, pH 7.2
2. 0.04 M sodium azide

3. 5×10^{-4} M DCIP
4. 0.05 M Sodium succinate
5. Sample to be assayed

Procedure:

The rection mixture (5 mL) contains 3.2 mL of assay buffer, 0.5 mL sodium azide, 0.5 mL Succinate and 0.3 mL of enzyme sample. The decrease in absorbance/minute is measured at 600 nm aginst blank containing all components except Succinate.

Calculation:

Succinate dehydrogenase (μM DCIP/min/mg of protein) $= \frac{\Delta A_{600}/\text{min}}{16.96} \times$ mg of protein

SDS-PAGE:

Reagents:

1. 1.5M Tris-HCl pH 8.8
2. 0.4% SDS.
3. 30:1 acrylamide :bisacrylamide
4. 0.5 M Tris-HCl pH 6.6
5. N, N', N, N' tertamethylenediamine.
6. 1% ammonium persulphate.
7. Coomassie brilliant blue (CBR) R-250.
8. Methanol.
9. Acetic acid.

Procedure:

Gel preparation:

The electrophoresis was carried out using slab type SDS-PAGE Model with 7.5% polyacrylamide gel. A 10% running gel (1.5M Tris-HCl pH 8.8, 0.4% SDS, 30:1 acrylamide :bisacrylamide), 5% stacking gel (0.5 M Tris-HCl pH 6.6, 0.4% SDS, 30:1 acrylamide : bis acrylamide) were prepared and polymerized chemically

using 20 µl N, N', N', N' tertamethylenediamine (Himedia), and 100 µl of 1% ammonium persulphate (High purity laboratory chemicals, Mumbai)). A marker of known molecular weight (SDS marker, Sigma-7H) was also loaded along with the samples. The apparatus was connected with constant electric current (100V) till the bromophenol blue (BPB) reached the bottom of the plate.

Staining and destaining of gel:

The gels were put into a container with staining solution-containing coomassie brilliant blue (CBR) R-250 dissolved in 50% methanol with 10% acetic acid and water. Gels were left in the staining solution for overnight and destained in 5% methanol, 7% acetic acid and water with shaking until the bands became visible above the background. Both staining and destaining steps were carried out while shaking.

APPENDIX II

20X PBS (Phosphate buffer saline)
Dissolve in this order in 1 liter of H_2O:
2.88 g Na_2HPO_4
160.00 g Nacl
4.00 g KH_2PO_4
4.00 g Kcl
Adjust to pH 7.2 with 10 M NaOH. Filter and store at room temperature.

0.2 M Phosphate buffer
21.8 g Na_2HPO_4
6.4 g NaH_2PO_4
Adjust to pH 7.0 with 0.1 N NaOH.

Mannitol assay buffer
0.3 M Mannitol
0.006 M KH_2PO_4
0.014 M K_2HPO_4
0.001 M KCl
0.005 M $MgCl_2$
Adjust to pH 7.2

0.04 M Sodium azide
Add 260 mg to the 100 mL distilled water.

0.05 M Sodium succinate
Add 1.351 g to the 100 mL distilled water.

DCIP (Dichlorophenol-indophenol)
Add 16.3 mg of DCIP to the 100 mL distilled water.

250 mM Phosphate buffer
2.00 g K_2HPO_4
0.495 g KH_2PO_4
Dissolve in 100 mL distilled water. Set pH 7.4

1.5 mM NADH
Dissolve 10.64 mg of NADH in 10 mL of distilled water.

7.5 mM OAA (oxaloacetate)
Dissolve 9.9 mg of OAA in 10 mL of distilled water.

Stacking Gel (For 5 ml):

30% Acrylamide, 0.8% Bis	0.65ml
4X Tris Cl/SDS pH 6.8	1.25ml
10% APS	0.025ml
TEMED	0.005ml
H_2O	3.05ml

Coomassie blue staining solution:
50% methanol (v/v)
0.05% (v/v) CBBR-250
10% acetic acid
40% water
Dissolve CBB-R in methanol. Add acetic acid and water. This solution can be stored at room temperature for 6 months.

Destaining Staining-
5% methanol
7% acetic acid
88% water
This solution can be stored for 1 month at room temperature.

Staining Gel (10%):

30% Acrylamide, 0.8% Bis	3.75ml
4X Tris Cl/SDS pH 8.8	3.75ml
10% APS	0.05ml
TEMED	0.01ml
H_2O	