CHARACTERIZATION OF THE SHORT-TERM EFFECTS OF PERINATAL ASPHYXIA AND SCREENING OF SOME ETHIOPIAN MEDICINAL PLANTS FOR THEIR PROBABLE PREVENTIVE ROLE IN RATS.

THESIS

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BY

EPHREM ENGIDAWORK

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ABBREVIATIONS

(1S, 3R)-ACPD	-	1S, 3R-1-aminocyclopentane-1,3-dicarboxylic acid
AMPA	-	alpha amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOSS	-	agonist operated calcium channels
ATP	-	adenosine tri phosphate
CBF	-	cerebral blood flow
CNQX	-	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	-	central nervous system
DMSO	-	dimethylsulfoxide
DNA	-	deoxyribonucleic acid
DOPAC	-	dihydroxyphenylacetic acid
EAA	-	excitatory amino acids
GABA	-	gamma amino butyric acid
HIE	-	hypoxic-ischemic encephalopathy
HPLC	-	high performance liquid chromatography
5-HIAA	-	5-hydroxy indole acetic acid
5-HT	-	5-hydroxy tryptamine
HVA	-	homo vanilic acid
IP_3	-	inositol-1,4,5-triphosphate
mGlRs	-	metabotropic glutamate receptors
MAO	-	mono amine oxidase
NBQX	-	2,3-dihydroxy-6-nitro-7-sulfamoyl benzo(f) quinoxaline
NMDA	-	n-methyl-d-aspartate
NOS	-	nitric oxide synthase
PH_i	-	intracellular pH
VSCC	-	voltage sensitive calcium channels

ABSTRACT

Perinatal asphyxia results from failure of normal respiratory gas exchange during or soon after labor and it remains an important cause of permanent neurological deficit in surviving infants. The most common features are hypoxia, hypercapnia, and metabolic acidosis. The present study was undertaken to further characterize the short-term effect of perinatal asphyxia and to investigate a possible preventive role of some Ethiopian medicinal plants and hypothermia in rats.

The effect of perinatal asphyxia on survival pattern, brain and heart pH, levels of amino acids, monoamines, and glycolytic intermediates was studied using in vivo microdialysis and ex vivo biochemistry. Perinatal asphyxia was induced by immersing fetus containing uterus horns, obtained by cesarean section from term pregnant rats, in a water bath at 37°C for different periods (0-23 min), according to a non-invasive model that largely mimics the conditions resulting in asphyxia during human labor (Bjelke et al., 1991; Andersson et al., 1992; Herrera-Marschitz et al., 1993). Subcutaneous levels of pyruvate, lactate, glutamate, and aspartate were monitored with microdialysis 80 min-8 days following delivery. In parallel experiments, pups were sacrificed 40 min after delivery and the brain and heart were removed to measure pH. In addition, pups were also sacrificed 80 min-8 days after delivery and the brain was removed to measure striatal levels of pyruvate, lactate, glutamate, aspartate, and monoamines.

At 37°C, a decrease in the rate of survival was first observed following asphyxic period longer than 16 min and no survival was observed after 22 min. pH decreased with the length of asphyxia. In control pups (cesarean delivered), brain pH was (7.3±0.01;N=6) and heart pH was (7.35±0.01; N=6). A significant decrease in pH was observed following 10-11 min and 5-6 min, in brain and heart respectively. After 80 min of delivery, a significant increase in the levels of all the measured compounds, in subcutaneous and brain tissues, were observed following exposure to mild asphyxia. However, the levels started to decline when asphyxia was prolonged. With increasing age, the levels of the measured compounds in mild asphyxic pups were almost similar as that of the control pups. Nonetheless, the time needed to recover depended upon how greatly the compound's metabolism was affected. Lactate being the most severely affected, much time was needed to reduce its level. Thus, changes in systemic pH, glycolytic intermediates, monoamines, and excitatory amino acids metabolism were

observed following perinatal asphyxia. In particular, subcutaneous level of lactate preceded: (a) a decrease in brain pH, (b) an increase in brain lactate level, (c) a decrease in the rate of survival, and probably (d) brain damage.

The possible protective effect of some herbal medicines was evaluated by injecting the extract subcutaneously or using as a bathing fluid and subjecting the pups to asphyxia at 37°C. Asphyxia induction at 30°C and 15°C was also carried out to evaluate the protective effect of hypothermia and to use it for comparison purpose. Survival was prolonged when asphyxia was induced under hypothermic condition. No survival was observed after 50 min and 140 min when asphyxia was induced at 30°C and 15°C respectively. Survival pattern after treatment with plant extracts did not show any significant difference compared to saline injected control group. Thus, hypothermia seems the only intervention that can provide good protective effect amongst the interventions so far evaluated.

However, with improvement in obstetric management, its role has been shown to be limited. As early as the 1930s, the cause of perinatal brain damage was intimately tied up with attitudes towards the use of sedatives, analgesics, and anesthetics during labor and delivery (Eastman, 1936). It was demonstrated that the excessive use of these agents caused "apnea neonatorum" which was thought to be the principal cause of cerebral injury (Schreiber, 1938) and almost all writers of the time supported this view and equated "asphyxia neonatorum" with "apnea neonatorum". Although this view was later shown to be unlikely (Myers, 1977), the tendency to consider birth apnea as a causative factor for cerebral injury dominated the 1940s and the 1950s. Hence, articles appeared in the 1940s strongly suggested a causal relationship between perinatal asphyxia and certain patterns of neuropathogenic changes in the brain. It was stated that the brain swelling and necrosis observed in newborns who died after cesarean delivery because of premature detachment of the placenta was due to asphyxia (Clifford, 1941). The injuries at birth were thought to be associated either to trauma to the head or to fetal systemic hypotension caused by asphyxia (Malamud, 1959,1963; Norman, 1969). It was believed that cerebral venous congestion causes the haemorrhagic infarction that often affects the brains of birth-injured babies (Schwartz, 1961). The congestion was attributed to the rapid passage of the fetal head from a zone of high pressure within the uterus to one of low pressure outside (Schwartz, 1961). The infarction of the cerebrum associated with birth injury was caused by fetal circulatory failure, generalized venous congestion, and cerebral venous stasis-thrombosis (Towbin, 1970). Thus, a number of causes have been proposed for perinatal brain damage of which perinatal asphyxia is one of the candidates.

Asphyxia is defined as suffocation with anoxia and increased carbondioxide. It arises from impairment of normal respiratory gas exchange with resulting hypoxia/ischemia, hypercapnia, and metabolic acidosis. The term perinatal asphyxia is often used to indicate an

impairment of gas exchange during or soon after labor (Nelson and Leviton, 1991; Martin and Nelson, 1993). The term hypoxic-ischemic or postasphyxial encephalopathy is often used to describe the illness thought to stem from such impairment. In most instances, during the perinatal period, hypoxemia and/or ischemia occur as a result of asphyxia (Hull and Dodd, 1991). When describing oxygen deprivation in human, the term asphyxia is used, because it is not known whether the insult is hypoxic, ischemic, or more probably a combination. Furthermore, regarding the fetus, the terms hypoxia and ischemia have been used interchangeably, because, the most common cause of hypoxia in the fetus is hypoperfusion or ischemia. Hypoxia can also cause ischemia, as it is capable of producing hypotension and reduced cardiac output. Thus, in asphyxia, the major additional feature is hypercapnia, which results in a number of other metabolic disorders, such as acidosis and physiological effects including cerebral vasodilatation (Volpe, 1987). Hypoxia and partial regional ischemia commonly occur together, therefore, it appears that the regional distribution of ischemia in the face of hypoxia is a major determinant of the relatively selective nature of perinatal asphyxial brain injury. Hence, this type of brain injury is referred to as hypoxic-ischemia.

Perinatal asphyxia can occur in the human fetus or neonate as an acute total asphyxial episode resulting from cord prolapse that leads to complete cessation of blood flow (Leech and Alvord, 1977), and as a prolonged partial asphyxial episode resulting from placental abruption that may occur during a long and complicated labor (Clifford, 1941). In order to understand the patterns of perinatal brain damage, two models in monkeys have been developed: *acute total asphyxia* (Ranck and Windle, 1959) and *prolonged partial asphyxia* (Brann and Myers, 1975; Myers, 1972, 1977). The first model that replicated acute total asphyxia caused a lesion affecting spinal cord, brainstem and thalamus without brain swelling. The second model that replicated prolonged partial asphyxia, however, produced a different pattern of cerebral injury

affecting mainly the cortical and subcortical structures with brain swelling (Myers, 1972). The reason for the different distribution of the lesions depends upon the redistribution of regional cerebral blood flow and the degree of neuronal maturation during asphyxia. In most cases, the total perinatal insult in humans most likely results from prolonged partial asphyxial episode, and sometimes from partial, combined with terminal acute asphyxial episode (Scott, 1976; Brann, 1986). Hence, fetal partial asphyxia of any cause, independent of fetal circulatory collapse and head compression, is believed to be the primary event that sets in motion a vicious cycle of brain swelling, leading to stasis of blood flow and, finally to cerebral necrosis (Brann and Myers, 1975).

Information about the specific effect of birth asphyxia on the fetus or neonates has been possible only since the development of new techniques for determining blood pH and blood gases. The introduction of risk scoring and assessment of fetal behavior has further improved the identification of the fetus at risk for asphyxia (Brann, 1986; Low et al., 1992). Thus, Apgar (1953) developed a scoring system to infer the occurrence of birth asphyxia and to quantify its severity from several indicators, such as: (i) type of breathing; (ii) heart rate; (iii) color of the skin; (iv) muscle tone; and (v) response to different sensory stimuli. Unfortunately none of these indicators is an accurate predictor of outcome, rather they are probably best used to indicate the need for active resuscitation (Hull and Dodd, 1991). Biochemical data such as umbilical pH and gas levels obtained soon after birth may be used to validate the judgement that the pathophysiological changes observed during birth are asphyxial in nature. But these putative markers of asphyxia do not always correlate well with one another.

Because of the poor predictive value of the traditional indicators, alternatives have been sought. This effort led to the identification of the abnormal neurological signs known as hypoxic-ischemic encephalopathy (HIE), that was used as an assessment of asphyxia (Sarnat

and Sarnat, 1976). HIE develops in the first few hours and days of life and is characterized by abnormalities of tone, feeding, level of consciousness, and in the more severe cases, seizures and finally coma with the need for ventilatory support. The postasphyxial encephalopathy is graded into mild (no seizure), moderate (seizures) and severe (coma). Those infants with mild encephalopathy have a uniformly good outcome, those with moderate encephalopathy have a 20-30% chance of severe handicap, and the majority of infants with severe encephalopathy die (Hull and Dodd, 1991). Hence, HIE has been found to be a much more accurate predictor of outcome (Robertson and Finer, 1985), however, the recent identification of a group of infants with typical encephalopathies (Hull and Dodd, 1991), without previous evidence of asphyxia cast some doubt on the casual relation between the two phenomena. Thus, there is still no reliable clinical indicator of birth asphyxia. Nevertheless, with the development of magnetic resonance spectroscopy, a potential independent indicator of brain asphyxial states has emerged (Martin and Nelson, 1993).

Several animal models have been developed to assess the role of asphyxia in mediating brain damage (see Raju, 1992). In the present thesis, the short-term effect of perinatal asphyxia and its prevention was studied in rat using a novel non-invasive model that largely mimics the conditions resulting in asphyxia during human labor (Bjelke et al., 1991; Andersson et al., 1992; Herrera-Marschitz et al., 1993).

1.2 PATHOPHYSIOLOGY OF ASPHYXIA

There are still major controversies related to timing, pathophysiology, and optimal management of perinatal asphyxial damage. Improved techniques for fetal assessment have focused on the intrauterine period as a major time of onset of neonatal neurologic diseases (Hill, 1991), which may arise from specific antepartum disorders (Brown et al., 1974; Low et al., 1992). Nevertheless, asphyxial insults caused by intrapartum distresses also play a crucial role in manifesting the clinical signs (Younkin, 1992). Most of the neuropathology associated with asphyxia is related to regional ischemic infarction and/or selective neuronal degeneration caused by a combination of reduced oxygen content of blood and regional reduction in cerebral blood flow (Volpe, 1987). Possible explanations for the selective vulnerability of neurons in specific locations within the brain may relate to a combination of regional circulatory, metabolic factors (e.g., localized differences in anaerobic glycolysis, energy requirements, lactate accumulation, calcium influx, free radical formation), as well as regional distribution of excitatory synapses.

The two most vital organs, brain and heart, receive oxygenated blood in preference to the other organs. The central nervous system is particularly vulnerable to the lack of oxygen due to: (i) a high rate of energy consumption to maintain cellular homeostasis, (ii) low reserves in substrates suitable for anaerobic energy metabolism (Hansen, 1985; Erecin'ska and Silver, 1989). The human infant possesses a number of adaptive mechanisms (e.g., redistribution of systemic blood flow, cerebrovascular autoregulation) to protect vital body functions during asphyxia. These adaptive mechanisms spare the brain and myocardium until the severity of asphyxia surpasses the threshold, and once exceeded, there will be a failure of energy supply to these vital organs. The resulting compromise to cardiac function may cause ischemic damage to already hypoxic tissues.

neuronal death in several diseases. This possibility was originally suggested by the work of Lucas and Newhouse (1957) who showed that systemic administration of glutamate to mice resulted in retinal degeneration. The subsequent studies of Olney (1969, 1986) linked neurotoxicity to activation of EAA receptors, and the term *excitotoxin* was coined. A further advance was the observation by Rothman (1984), that release of EAAs mediated cell death in anoxic hippocampal cultures, and the demonstration that antagonists could ameliorate ischemic damage in the hippocampus. The development of selective antagonists has facilitated the identification of excitatory amino acids as major transmitters in the CNS, and provided the means to test the hypothesis that the toxicity of EAAs is a consequence of excessive activation of EAA receptors. According to the excitotoxic hypothesis extracellular concentration of glutamate and aspartate rise markedly, probably because of enhanced release and inhibited reuptake (Seisjö, 1981, 1988; Vannucci, 1992,).

Glutamate is released from nerve terminals by two processes. The first is the classical exocytotic, Ca^{2+} dependent pathway, which utilizes amino acids stored in intrasynaptic synaptogamine-dependent vesicles; the second is the Ca^{2+} independent efflux which occurs via reversal of the high affinity transporter from the cytoplasmic pool. The former, like any stimulus coupled secretory events requires ATP, whereas the latter is stimulated when the energy level is decreased and ion gradients across the plasma membrane is reduced. This reversal transport is the main route of efflux during pathological conditions that result in low ATP (Erecin'ska and Silver, 1990). Glial cells in contrast to nerve terminals, utilize only the second mechanism of glutamate release. Since the transmembrane Na^{+} gradient and membrane potentials (driving forces for glutamate accumulation) are larger in glia than neurons the astrocytic transporters would be expected to contribute more to reversal transport (Erecin'ska and Silver, 1989).

On the basis of pharmacological, electrophysiological, and biochemical studies, glutamate receptors can be classified into two types; ionotropic and metabotropic receptors (Watkins and Evans, 1981, Beal, 1992). The ionotropic receptors contain integral, cation-specific ion channels, whereas the metabotropic receptors are coupled to G proteins and modulate the production of intracellular messengers. Ionotropic receptors can further be divided into NMDA, AMPA, and Kainate receptors according to their agonist profiles. There are at least eight subtypes of metabotropic glutamate receptors (mGluRs) (mGluR1 through mGluR8) as well as splice variants of three types and are activated by quisqualate, ibotenate, glutamate, 1S, 3R-ACPD, and L-amino phophonobuytric acid (Nakanashi, 1992; Pin and Duvoisin, 1995). The eight receptor subtypes can be further subdivided in to three subgroups on the basis of their signal transduction mechanism, sequence homology, and pharmacological properties. Group I (mGluR1 and mGluR5, and splice variants); Group II (mGluR2 and mGluR3); and Group III (mGluR4, mGluR6, mGlu7, and mGluR8). Group I mGluRs are coupled to polyphosphoinositide hydrolysis and subsequent calcium release and also induce arachidonic acid release. In contrast, Group II and III mGluRs are negatively coupled to adenyl cyclase (Nakanashi, 1992; Holmes et al., 1996). The physiological role of mGluRs is not well understood, but recent evidence has indicated that some of them could be used as a target for the treatment of human neuropathologies (Ambrosini et al., 1995; Copani et al., 1995).

There are two proposed mechanisms for glutamate toxicity (Vannucci, 1990; Choi, 1992):

(i) Acute neuronal swelling (Osmotic lysis): occurs within minutes of exposure of onset and believed to be due to the action of glutamate on kainate and AMPA receptors causing excitation by increasing Na^+ conductance. Since this channel allows Na^+ influx to occur along a steep electrochemical gradient, its opening forces Cl^- to enter via normal or activated anion channel.

The entry of Na^+ and Cl^- increases cell osmolality, leading to accumulation of water into the cell. Subcellular edema ensues, which if severe enough leads to cell swelling and lysis.

(ii) Delayed cell degeneration: is marked by delayed neuronal disintegration that occurs over a period of hours. Believed to occur via NMDA receptors, by which glutamate gates a channel that permits calcium influx. Calcium, in turn, sets in motion a cascade of events that culminate in cell death.

Although most available evidences suggest that excitotoxicity is receptor mediated, it is possible that some injury might occur independent of glutamate receptor activation. For example, carrier mediated uptake of high concentrations of extracellular glutamate might contribute to the movement of sodium (by co-transport) and water (by osmolar shift), contributing to cell swelling and eventual calcium overload (Choi, 1992).

b) Disturbance of Calcium homeostasis

Calcium is intimately involved as a cofactor in numerous biochemical reactions, thereby acting as a regulator of cellular metabolic homeostasis. Therefore, it is not surprising that a disruption of calcium homeostasis has a wide-ranging effects on neuronal metabolism and function. The mechanism by which altered calcium balance occurring during cerebral hypoxia-ischemia threatens the cell continues to be elucidated but undoubtedly is related to disturbances in those biochemical reactions subserved by the cation. The concentration of this cation in the cell is tightly regulated because of its strategic role in metabolic regulation. Indeed almost all intracellular calcium is bound to subcellular structures and the free cytosolic concentration is closely regulated by fluxes across the plasma membrane. Specific ion channels for calcium exist in all cells, which either are voltage sensitive calcium channel (VSCC), or agonist operated calcium channel (AOCC) of the NMDA type.

Asphyxia causes increased calcium influx into cells because depolarization and transmitter release open VSCC and AOCC. In addition to transmembrane calcium influx, inositol 1,4,5-triphosphate (IP_3) mediated intracellular mobilization of calcium induced by metabotropic glutamate receptor activation also increases intracellular calcium. Furthermore, energy failure after an asphyxial insult prevents ATP-dependent calcium extrusion, therefore, loss of ATP combined with anoxia prevents the mitochondria and endoplasmic reticulum from sequestering calcium leading to a further increase in intracellular free calcium. Thus, the increase in intracellular free calcium produced in such a way, severely compromises the viability of the neuron in the reperfusion period, when the oxygen supply is restored.

The calcium ion is an important regulator of cellular homeostasis. Elevated concentration of intracellular calcium activates lipases, proteases, and endonucleases which disrupt the structural integrity of cell membranes. Moreover, high concentration of intracellular calcium results in uncoupling of oxidative phosphorylation within mitochondria. Thus, excessive intracellular calcium accumulation causes membrane disintegration and neuronal death. Calcium activation of calpain I & II leads to alterations in the cytoskeleton. The activation of phospholipase A_2 leads to breakdown of phospholipid membranes that results in liberation of fatty acids, such as arachidonic acid. Arachidonic acid is a substrate of cyclooxygenases and lipoxygenases that produce free radicals and vasoactive species. Activation of a specific protease converts xanthine dehydrogenase to xanthine oxidase which is a rich enzymatic source of free radicals. Activation of endonucleases leads to degradation of DNA and activation of nitric oxide synthase (NOS) leads to a burst of free radicals production. Nitric oxide formed by NOS reacts with superoxide anion to give peroxynitrite anion which subsequently decomposes to toxic hydroxyl radicals in the presence of an acid (Volpe, 1987; Vannucci, 1990; Seisjö et al., 1993).

c) Oxidative stress

The oxidative stress hypothesis infers an imbalance between the formation of cellular oxidants and the antioxidative processes. It occurs due to the excessive formation of hydrogen peroxide and oxygen-derived free radicals. A free radical is a compound that has a lone electron in its outer orbital. This electron configuration confers to the compound an unusual reactivity. As a result, free radicals have a tendency to attack neighbouring molecules in reactions that can be propagated in an autocatalytic manner. The important production sites of such free radicals (e.g. the superoxide ($.O_2^-$) and hydroxyl (OH .) species) are the mitochondrial respiratory chain and the sequences catalyzed by cyclooxygnases and lipoxygenases. Free radicals can be produced within the mitochondria when cytochrome oxidase is not fully saturated with oxygen, thereby liberating free radicals at more proximal site. These free radicals can not be consumed further and leak out into the cytoplasm. Free radicals are also formed during autoxidation of many compounds (e.g., catecholamines) and in xanthine oxidase reaction. NO produced in the brain as a consequence of glutamate NMDA receptor activation is a membrane diffusible free radical (D'Souza and Slatter, 1994). It can also give rise to a burst of free radical production by reacting with superoxide anion.

There have been increasing arguments that suggest, the reoxygenation or reperfusion phase following an ischemic insult may aggravate tissue injury by yielding reactive oxygen species. Recent investigations have provided evidence for the formation of these species in the vulnerable regions of the brain (Lancelot et al., 1995). Free radicals can cause tissue injury, primarily by leading to peroxidation and subsequent fragmentation of polyenoic fatty acids of membrane phospholipids. This peroxidation afterward causes enhanced rise in the level of aldehydic lipid peroxidation products such as 4-hydroxynou-2-enal and molondialdehyde which act as second toxic messengers. The membrane fragmentation caused by lipid peroxidation in

turn may lead to cross linking of proteins with inactivation of critical enzymes and transport molecules (Siesjö, 1988). Transition metals mediated radical reactions may convert single stranded nicks of DNA, accumulated during the insult as a result of calcium activated nucleases, to lethal double stranded nicks (Krause et al., 1988). Some free radicals like NO can cause disturbance in mitochondrial energy metabolism. NO inhibits several enzymes including NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), and cytochrome oxidase (complex IV) of the mitochondrial electron transport (Gerlach et al., 1995). Inhibition of these enzymes can lead also to production of additional free radicals making the event a vicious cycle.

Although the exact mechanism of lipid peroxidation is unknown, it is well established that the presence of a transition metal is required as a catalyst (Aust et al., 1985). At least two transition metals, copper and iron, are found in sufficient quantities in the body to act potentially as catalysts for initiation of lipid peroxidation. Since copper is made unavailable by the histidine component of proteins, the most likely candidate is iron. The brain has an abundant stores of iron, as do all cells. Most of this iron is in haemenzymes and in storage proteins such as ferritin, or in transport proteins such as transferrin. In these forms, the iron is unable to act as a catalyst for oxygen radical reaction. As iron is stored in an oxidized form in the ferritin molecule, reduction to the ferrous form is required to cause its release. It has been shown that superoxide promotes the release of iron from ferritin (Thomas et al., 1985). Since oxygen is essential for superoxide production, iron is released only during reperfusion. Thus, iron released during reperfusion is believed to cause iron-dependent lipid peroxidation (Krause et al., 1988).

d) **Metabolic acidosis**

Under normal circumstances, glucose is virtually the exclusive substrate for ATP production in the brain. Within seconds of the onset of hypoxia-ischemia, oxygen is depleted and oxidative phosphorylation ceases. Under these conditions, the only source of ATP production is

anaerobic glycolysis. The shift to anaerobic metabolism produces large increase in lactate and NADH and a decrease in intracellular pH. Increased lactate level is inversely related to neuronal recovery (Rehncrona et al, 1980). Although the mechanism of this relationship is not clear, marked acidosis during hypoxia-ischemia may prevent or retard recovery by different mechanisms (Seisjö, 1988; Seisjö et al., 1993): (i) acidosis causes swelling of vascular cells and predisposes to sludge and other phenomena, decreasing the quality of reperfusion and promoting inflammatory reactions in the microvasculatures; (ii) extracellular acidosis slows down Na^+ coupled H^+ extrusion that prevents or delays normalization of pH_i; (iii) Since acidosis retards oxidative phosphorylation, ATP production is reduced. This leads to a precarious situation in which too little energy is formed by the acidotic cell, which in the absence of an energy source to build up a Na^+ gradient, can not correct the acidosis. On the other hand acidotic cells, with preserved ATP production could attempt to restore pH_i at the expense of their volume regulation. The expected result of such a state is progressive edema; (iv) acidosis prolongs the calcium transient into the recovery period, triggering calcium-induced damage. This probably, is due to outcompetition of Ca^{2+} by H^+ for intracellular binding sites or enhanced regulation of pH_i by Na^+/H^+ exchange that sets the stage for reversed Na^+/Ca^{2+}, which increases the influx of calcium; (v) acidosis may lead to enhanced free radical production either by converting $.O_2^-$ to its hydrated, more lipid soluble and more pro-oxidant form, hydroperoxy radical ($H.O_2$), or by releasing pro-oxidant iron from protein bindings in transferrin and ferritin, favouring chelation of iron to low-molecular weight compounds which are pro-oxidant. Furthermore, acidosis may favour free radical production by virtue of its role in the sequence of reactions leading to production of NO and its toxic products. NO in the presence of $.O_2^-$ gives peroxynitrite, which later decomposes to yield the extremely toxic .OH species. Acidosis

accelerate this latter reaction. Hence, intracellular acidosis exerts further its deleterious effects by accelerating other reoxygenation or reperfusion mechanisms.

1.3 MANAGEMENT OF PERINATAL ASPHYXIA

At present, there is no consensus for the management of perinatal asphyxia. Insults are likely to induce cell death by several simultaneous injury processes. Interventions directed at interrupting a given mechanism may produce a change in cell death only if the mechanism is a lead mechanism bearing responsibility for death. Otherwise, its contribution will be masked by other processes that are capable of inducing death sooner. Brain damage following asphyxia can be ameliorated by: (i) prompt resuscitation, (ii) hypothermia, (iii) drugs.

1.3.1 Resuscitation and other supportive therapies

Resuscitation of the newborn infant is one of the major pediatric emergencies. The whole purpose of resuscitation is to give adequate ventilatory support and to maintain an adequate circulation. Current resuscitation methods include: (i) providing oxygen and removing carbondioxide by positive pressure ventilation; (ii) maintaining the circulation by external cardiac massage; (iii) using a volume expander to combat shock; (iv) and infrequently, if the infant fails to respond, using cardiotonic agent and providing alkali for buffering the excess acid produced during anaerobic metabolism. An intact circulation is of major importance in the ability to survive asphyxia, since it is able to redistribute lactate and hydrogen ion to tissues still being perfused and thus may provide a means of buffering cells during asphyxia. By appropriate intervention with adequate resuscitative measures the severely asphyxiated infant can be saved and brain damage prevented or minimized. Brain injury occurs when prolonged asphyxia overwhelms the compensatory mechanisms previously described. The damage may be severe

and manifested early in the form of seizure disorder, cerebral palsy, or later on as profound developmental retardation.

Efforts to minimize damage are directed at managing or preventing problems of inadequate cerebral blood flow, cerebral edema, and intracerebral haemorrhage. There is no generally accepted approach to manage cerebral edema in the neonate (Vannucci, 1990). In the past, therapies directed towards reducing cerebral edema included the use of hyperventilation, dehydration, glucocorticoids, high dose barbiturates, and osmotic diuretics, either alone or in combination. However, the effectiveness of any of these treatments has not been conclusively demonstrated (Fisher and Paton, 1986; Vannucci, 1990). Neonatal seizure can be managed by correcting any underlying metabolic derangement or structural lesion of the brain if present, and then early control of convulsive activity with antiepileptic medications. Once it has been established that the metabolic derangement is not contributing to seizure activity in the asphyxiated newborn infant, an anticonvulsant medication is used in an attempt to control repetitive or continuous seizure activity (Vannucci, 1990).

1.3.2 Hypothermia

The idea, "cold bath for baby" is not entirely novel. The custom of bathing neonates in cold water was world wide among primitive people. It was suggested a number of years ago that man may have discovered during the glacial epoch that when, apparently dead babies were placed in cold streams they sometimes began breathing (Miller, 1957).

Hypothermia has been long known to increase brain tolerance to a variety of insults. Experimental studies have demonstrated that hypothermia provides protection for animals exposed to longer time of asphyxia (Miller, 1971). Moderate hypothermia is effective in significantly increasing survival during hypoxia-ischemia, however, it provides only partial

protection from neuropathologic damages. Profound hypothermia on the other hand, ameliorates the systemic and cerebral metabolic derangements and prevents the neuropathologic changes caused by hypoxic-ishemic injury in neonatal rats and newborn dogs (Young et al., 1983; Mujsce et al., 1990). It has been demonstrated that low intraischemic brain temperatures can protect brain neurons in rats subjected to transient forebrain ischemia (Busto et al., 1987) and postischemic hypothermia of only a few degrees can also have a significant protective effect (Buchan and Pulsinelli, 1990). Further it has been shown that rats can maintain a constant plasma pH under hypothermic condition (Majewska et al., 1966).

The benefit of cooling during asphyxia is mainly related to the reduction in the over-all oxygen requirements. Animals at 37°C use more oxygen than they do at 15°C (Miller, 1971). Lactate production during anoxia is directly proportional to tissue metabolism. In warm animals, there is a rise in lactic acid accumulation and blood pH falls at a rapid rate. Since low pH interferes with many enzymatic processes, cardiac contractility, and resuscitation of an asphyxiated neonate (Miller, 1971), the importance of reducing body temperature during asphyxia appears to be obvious.

1.3.3 Drugs

Although it has proved possible to show that certain drugs exert a neuroprotective action at cellular, biochemical and functional levels, in various experimental models of neurodegeneratve diseases, so far, it has not yet been possible to demonstrate an unequivocal neuroprotective effect for any drug in clinical studies (Gerlach et al., 1995). Drugs that are used as supportive therapy are discussed under resuscitation. Under this heading, drugs that are under intense investigation for management of perinatal asphyxia will be considered.

a) Excitatory amino acids antagonists

As discussed previously, recent studies have yielded compelling evidence that excessive exposure of neurons to glutamate or other EAAs may result in neuronal death. Asphyxia causes excessive glutamate release and it is a major cause of postasphyxial neuronal damage (Levene, 1992). Given these premises, it is rational to search for pharmacological agents that would either inhibit glutamate release or block its postsynaptic action. Limited studies have been performed to assess the neuroprotective effect of inhibitors of glutamate release from the nerve terminal. Pre-treatment with low doses of baclofen, a selective agonist of $GABA_B$ receptors and inhibitor of EAA release, has not shown any protective effect in the four-vessel occlusion model of cerebral ischemia (Obrenovitch and Richards, 1995). Receptor antagonists in contrast, have undergone extensive study in experimental animals (Scatton et al., 1991; Barks and Silverstein, 1992; D'Souza and Slater, 1994; Estevaz et al., 1995). Of these drugs, NMDA antagonists have received the most attention. Available compounds include (i) competitive recognition site antagonists (e.g., CGS 19755, CGP 37849, LY 233053); (ii) channel blockers or those associate with the PCP site (e.g., phencyclidine, ketamine, dextromethorphan, and dizocilpine), (iii) modulatory site antagonists such as; those antagonize the coagonist action of glycine (e.g., HA-966, cycloleucine, L-687414, and L-701324) and those acting by blocking the potentiating effect of polyamines (e.g., SL 820715, ifenprodil). There are a number of studies that suggest, NMDA antagonists given before or after experimental hypoxia-ischemia protect the developing brain (McDonald et al., 1989, 1990). Noncompetitive AMPA/Kainate receptor blockers, including NBQX, CNQX, and GYK-152466 have been shown to be effective in animal models of asphyxia (Herrera-Marschitz et al., 1993) and global and focal brain ischemia (D'Souza and Slater, 1994).

The discovery of mGluRs negatively coupled to adenylate cyclase opened a new avenue of therapy for acute and chronic neurodegenerative disorders. Recent studies strongly encourage the development of compounds that are highly selective for these neuroprotective mGluR subtypes (Ambrosini et al., 1995; Copani et al., 1995), since they may be a potential target for specific drugs in the experimental therapy of human neuropathologies.

So far, animal studies have produced data which suggest that non-competitive NMDA and AMPA/Kainate receptor antagonists may protect against cellular damage. Although there are no clinical data on this group of drugs, there are suggestions that their use may represent an important strategy in the future management of the asphyxiated newborn infant (Levene, 1992; Herrera-Marshitz et al., 1993). Further a number of NMDA antagonists such as SL-820715, CGS-19755, and dizocilipine are now in the early phases of clinical trials and their use as a remedy will be decided in the next few years.

In considering treatment with EAA antagonists, an important issue that must be acknowledged is their potential detrimental effect in the developing brain. Since EAA play critical roles in normal synaptic maturation, EAA blockade could disrupt normal developmental processes (Teng et al., 1996). Therefore, the adverse impact of EAA antagonists will require careful evaluation before such drugs come into clinical use. Drugs that block excessive release or promote enhanced reuptake might provide neuroprotection more selectively than receptor antagonists.

b) Calcium channel blockers

Because of the strategic role of calcium in the regulation of cell metabolism, treatment with calcium antagonists in the early phase after cerebral hypoxia-ischemia has been hypothesized to halt intracellular calcium ion accumulation thereby preventing the cascade of

events leading to ultimate neuronal destruction. Thus, different calcium channel blockers have undergone thorough investigation. Of the numerous calcium channel blockers, the dihydropyridine calcium channel blockers; flunarizine and nimodipine appear most efficacious in reducing the hypoxic-ischemic brain damage in adult animals. Although studies in newborn animals are limited, available results indicate that nimodipine and flunarizine may improve the neurological outcome in asphyxiated newborns (Laura et al., 1987; Gunn et al., 1989). Other studies have demonstrated that diphenylpiperazine calcium channel blockers can provide good neuroprotective effect. Lifarizine (RS-87476), has a broad neuroprotective profile in both global and focal ischemia (Alps, 1992). Another derivative of diphenylpiperazine, Lomerizine (KB-2796) has also shown to be a drug with proven protective effects against cerebral ischemia, both in vitro and in vivo (Hara et al., 1993; Yamashita et al, 1993). Possible mechanisms for calcium channel blocker's neuroprotective effects include a direct effect on cells to prevent calcium entry, selective down-modulation of voltage-gated Na^+ channels (Urenjack and Obrenovitch, 1996), the prevention of postischemic hypoperfusion, or an anticonvulsant activity.

Calcium channel blockers are currently undergoing clinical trials in patients with stroke and cardiac arrest, as well as in newborn infants who have sustained myocardial injury or arrhythmia, clinical investigations of such agents to protect against cerebral hypoxia-ischemia is forthcoming in the near future.

c) Sodium channel blockers

Voltage-gated Na^+ channels are responsible for initiation and conduction of neuronal action potential and therefore play a fundamental role in the normal function of the nervous system. Na^+ channels can potentially influence neurotransmitter release from presynaptic vesicles in nerve terminals. Because of this property, they play a role in glutamate toxicity. Owing to

these roles of Na^+ channels, recent reports demonstrate that selective down-modulation of voltage-gated Na^+ channels is a rational and effective approach to protect brain tissue in conditions associated with ischemia (Urenjack and Obrenovitch, 1996). Drugs which fall under this category include calcium channel blockers with action on Na^+ channels (flunarizine, lomerizine), lamotrigine and its structural analogues such as BW-1003C87 and BW-619C89.

d) Antioxidants and free radical scavengers

Oxygen free radicals generated during or after hypoxia-ischemia in several ways initiate and perpetuate chain reactions which ultimately lead to membrane fragmentation, the peroxidation of unsaturated fatty acids being a prominent example. A complex system of antioxidant defenses preserves the balance between oxidant production and removal. This system comprises enzymatic defenses such as superoxide dismutase, catalase, and glutathione peroxidase; and low molecular weight scavengers, such as vitamin C and E and glutathione. When the balance is upset by either excess radical production or a defective defense mechanism, oxidative stress and tissue injury can occur.

The therapeutic use of oxygen radical scavengers is under active investigation, particularly in relation to inflammatory and ischemic diseases. Administration of specific enzymes has been one therapeutic approach to the early destruction of oxygen derived free radicals. The enzymes suffer from short plasma half lives, do not easily penetrate into intracellular sites where free radicals are produced, and do not cross the blood brain barrier. Thus, the initial hopes for enzyme therapy have not been realized. Nonetheless, recently by modifying enzyme molecules a dramatic effect has been observed (Rosenberg et al., 1989). An alternative approach is the search for inhibitors of free radical generation and scavengers of free radicals. Low molecular weight scavengers have better accessibility and are receiving

considerable attention, however, their therapeutic benefit in various experimental models is not promising (Gerlach et al., 1995). Drugs that inhibit oxidant production have shown to be potentially efficacious in favourably influencing the outcome of hypoxic-ischemic damage. In immature rats, allopurinol, a xanthine oxidase inhibitor, has been shown to be beneficial in reducing the severity of perinatal hypoxic-ischemic brain damage (Palmer et al., 1990). This effect could be mediated presumably by inhibition of xanthine oxidase activity or via its role as a radical scavenger. Pre-treatment with indomethacin, a cyclooxygenase inhibitor, block the generation of superoxide anion which is thought to be responsible for the delayed postasphyxial hypoperfusion (Pourcyrous et al., 1990). Transition metals play an important role in radical reactions. Hypoxia may decrease intracellular pH through anaerobic glycolysis and thus, trigger free iron mobilization (Qi et al., 1995), so another approach is the administration of chelators such as desferroxamine. Diet is also an important factor, particularly in the neonatal period when intakes of micronutrients may be suboptimal, this could result in deficiency of antioxidant vitamins or decreased glutathione and glutathione peroxidase activities.

e) Herbal medicines

Before the advent of modern medicine, all nations and races had some kind of traditional medicine (folk medicine) on which they relied for their day-to-day health care. The knowledge about medicinal plants and their role in treatment of diseases is as old as man himself. Medicinal plants and substances derived from them have always played an important role in therapy within traditional health care systems globally. Many therapeutic regimens and practices, applied by traditional healers, may appear magical or mystical to an observer, trained within the modern scientific school of medicine. Nevertheless, it seems to be a fact that those regimens often have the intended effect. Sometimes these effects are referred to as placebo. There is, however,

agreement on that, behind the superficially mystical therapeutic regimens of many traditional healers, there is a nucleus of rationality and objective truth which may be verifiable and reproducible. This recognition implies that doors have opened towards a close collaboration between traditional herbalists, who have access to extensive empirical knowledge about the medicinal use of plants, and modern scientists, who may analyze this knowledge and separate the useful from those without effect, or even harmful ones.

In Ethiopia, although this practice is thought to have Arabic and Hebrew influence, the trend is by large considered to be indigenous, since the use of plants in religious ceremonies, as well as for magic and medicinal purposes is very common and wide spread that dates from ancient times. Today, the vast majority of the Ethiopian people rely on traditional medicine, which uses herbal preparations and other natural products, for the maintenance of health. This is because, the economic situation does not allow most of the people to have access to modern health care services. Ethiopia is endowed with a large number of plants well known to and widely used by many practitioners of traditional medicine. However, it is only very recently that the role of traditional medicine as well as other related practices, using crude plant parts and products, has been seriously considered by modern investigators.

Although there are no traditional herbs used to treat the asphyxiated newborn per se, there are many plants used to facilitate the birth process (Tadesse and Demissew, 1992; Abbinik, 1995). Under normal circumstances the process of birth is an asphyxial stress that sets a profound alteration of biochemical and physiological homeostasis. This alteration is not well tolerated if the brain is already stressed or if there is a prolonged and severe labor. So it will be logical to assume that plants which are claimed to facilitate the birth process may prevent perinatal asphyxia. Therefore, amongst them, *Clerodendron myricoides L.*

(Verbenaceae) and *Cardiospermium helicacabum L.* (Sapindaceae) which are believed in folklore medicine to have effect on the birth process were chosen for the pilot experiment. Further plants which are used to reduce perinatal mortality and morbidity and those that have possible hypothermic effect may provide protective effect. From those plants, *Heliotropium longifolium* (Boraginaceae) (Hedberg and Staugård, 1989) and *Teclea nobilis D.* (Rutaceae) (Mascolo et al., 1988) were screened for their probable protective effect.

2. OBJECTIVES OF THE STUDY

2.1 General objectives

Perinatal asphyxia is thought to be responsible or a concurrent factor for the development of behavioral and neurological deficits with onset at different stages of life. The cause of these deficits might be biochemical changes that occurred during the insult or shortly after reoxygenation or reperfusion. Therefore, it is interesting to assess the neurological status and monitor the levels of several neurochemicals and glycolytic intermediates, in order to further characterize the short-term effect of perinatal asphyxia. In addition, in the effort to develop a drug that provides full protective effect the probable preventive role of some Ethiopian medicinal plants and the protective effect of hypothermia are investigated.

2.2 Specific objectives

2.2.1 To study the behavioral changes that asphyxia can bring about on rat pups.

2.2.2 To measure the pH of the rat pup brain and heart after induction of asphyxia.

2.2.3 To analyze changes in the levels of glycolytic intermediates, such as pyruvate and lactate, and putative neurotransmitters such as glutamate and aspartate in subcutaneous and brain tissue after induction of asphyxia.

2.2.4 To analyze changes in the levels of monoamine transmitters such as dopamine and their metabolites in brain tissue after induction of asphyxia.

2.2.5 To evaluate the protective effect of hypothermia in this model of asphyxia.

2.2.6 To screen the possible preventive role of some herbal medicines against the consequences of perinatal asphyxia.

3. MATERIALS AND METHODS

Pregnant Sprague-Dawely rats weighing 400-450 gm (Alab, Stockholm, Sweden) with access to food pellets and water ad libtum and maintained in a temperature-controlled environment on a regular 12 h light/dark cycle were used for the experiment. In addition, Sprague-Dawely rats purchased from B&K, Universal, AB, Sollentuna, Sweden (Alab, Medical faculty, Addis Ababa) were also used. The gestational age of the rats was determined by (i) clinical palpation and (ii) behavioral observation (e.g., nest building, lying over the abdomen, starting delivery). Only rats in their last day of gestation were used for the experiment.

3.1 Induction of perinatal asphyxia.

Asphyxia was induced in rat pups delivered by cesarean section (Bjelke et al., 1991; Andersson et al., 1992; Herrera-Marschitz et al., 1993). Time-mated female rats (weighing 400-450gm), within the last day of gestation were anesthetised with ether and hysterectomized. Immediately after hysterectomy some pups were delivered as cesarean controls, thereafter the uterus, still containing the remaining fetuses, was placed in a 15°C, 30°C, and 37°C water bath for various periods of time to induce asphyxia. Following asphyxia, the uterus horns were rapidly removed from the water bath, and the pups were delivered and stimulated to breath. The umbilical cord was ligated, and the pups were left to recover on a heating pad. The time of asphyxia was measured from the time when the blood circulation to the uterus was cut off until the pups were taken out of the water bath. Some rats were let to deliver spontaneously and their pups were used as spontaneously-delivered controls. Some pups that remained alive during the experiment were given to surrogate mothers until needed for another experiment. In addition,

rats treated with saline and herbal extract were subjected to asphyxia in a similar manner at $37^{\circ}C$.

3.2 Behavioral observation.

Short-term effect of perinatal asphyxia was monitored by direct observation in 40-80 min period following delivery. A behavioral scale was developed to assess the general condition of the newborn pups that may be used as APGAR score in animal model. The parameters include: (i) survival rate, (ii) respiratory frequency, (iii) gasping, (iv) vocalization, (v) color of the skin, and (vi) spontaneous movements. A scale was used to score the intensity of movement as follows: (0) Akinesia and rigidity (mainly on the hind legs); (1) movement of one of the following body structures: front legs, hind legs or head alone (2) Movement of two of the body structures; (3) movement of all body structures; (4) Intensive movements shown by wriggling.

3.3 pH measurement in tissue.

Following 40 minutes after delivery control and asphyxic pups were killed by decapitation. The brain and heart were dissected out and stored at $-80^{\circ}C$ pending measurement. Later, pH determination was performed by inserting the tip of a pH electrode of 2 mm diameter (TFK 325/HC; pH 320 set, WTW, Wissen-schaftilch-Technische-Werkstaten, GmbH, Wein, Austria) into the whole tissue kept at $4^{\circ}C$ in an ice bath. Measurements were done in triplicate with pepsin-distilled water rinsing cycles and the means of each triplicate were taken as the measured value.

3.4 In vivo Microdialysis.

Eighty minutes, 24 hrs., 48 hrs., 4 days and eight days following delivery, a microdialysis probe (CMA 20, CMA/Microdialysis AB, Stockholm, Sweden), (Dialysis membrane length = 4 mm, diameter = 0,5 mm) was implanted subcutaneously into the dorsal region, with a caudo-rostral orientation, parallel to the spinal cord, of control and asphyxic pups. Microdialysis probe was perfused with Ringer solution (NaCl 147 mmol /lit, KCl 4 mmol/lit, $CaCl_2.2H_2O$ 2,3 mmol/lit, pH 6, Apotek Bolaget, Sweden), at a constant flow rate of 2μl/min, maintained with a microdia-lysis pump (CMA 100, CMA/Microdialysis AB). The pH of the perfusion medium was checked and adjusted to 7.2-7.3. After three 20-min samples, the microdialysis probe was removed, the wound was sealed with a drop of acryl blue (Histoacryl^R), and the pups were sacrificed. The samples were split in 10 μl aliquots and directly injected into high performance liquid chromatography (HPLC) systems, coupled to a fluorometric or UV detectors for analyzing glutamate and aspartate, or pyruvate and lactate, respectively.

3.5 Tissue preparation.

Eighty minutes, 24 hrs., 48 hrs., 4 days and 8 days old control and asphyxic rat pups were killed by decapitation and the brain was rapidly removed and stored in dry ice. Thereafter, coronal 400 mm sections were cut in a cryostat and punch samples (0.5-1 mm in diameter) were taken from the striatum. The samples were then stored in previously weighted 1.5 ml Eppendorf tubes at -80^{0}C until analysis. At time of analysis, 0.1 ml perchloric acid (PCA) (0.1M) was added to each tube, and then the tissue was sonicated until dissolved (Sonicator, Heat-systems Ultrasonics, Inc., Farmigdale, N:Y., USA). The sonicated tissue was centrifuged (Bergman centrifuge, CS-6R, USA) for 20 minutes at 5°C (3000 rpm). The aliquots were then injected directly to various HPLC systems coupled to electrochemical, UV, or fluorometric detectors to analyze monoamines, amino acids and glycolytic intermediates, respectively.

3.6. In vitro recovery.

The 4 mm microdialysis probes were tested for in vitro recovery of glutamate, aspartate, pyruvate, and lactate. This was done by perfusing the probe in a 2 ml vial containing a known concentration of glutamate (2.5 μM), aspartate (2.5 μM), pyruvate (25 μM), and lactate (250 μM) dissolved in Ringer at room temperature. Three 20 μl perfusate samples and one 20 μl external medium samples before and after perfusion were taken. Then, 10 μl of the aliquot was injected into an HPLC system connected to various detectors.

3.7. Biochemical analysis.

3.7.1 Glutamate and aspartate.

Glutamate and aspartate were determined by HPLC system with precolumn derivatization using O-phthaldehyde and mercaptoethanol as reagents and fluoroimetric detection. Briefly, 3 (10) μl of the reagent (0.4 M borate, 0.04 M O-phthaldehyde, 0.4 M 2-mercaptoethanol, pH 10.4) was added to 2 (10) μl of the sample obtained from the brain and subcutaneous tissue respectively. After a 60s reaction period at 7°C in a microsampler, 4 (15) μl of the derivatized aliquot was injected into a column prepacked with Biophase ODS 5 μm particles (Knauer, Berlin, Germany). The elution with 0.1M sodium acetate, 8% methanol (adjusted to pH 6.95), and 1.5% of tetrahydrofuran, was performed with an SP 8800-020 pump (Spectra-Physics, San Jose, CA, USA) equipped with a two way valve at a flow rate of 1ml/min. A linear gradient changing to 100% methanol over 2 min and returning to 100% eluent 2 min later was used to rapidly clean the column after elution of aspartate and glutamate. The fluorometric detector was an F1000 (Hitachi, Tokyo, Japan) with excitation wavelength set at 370 nm and emission cut-off filter set at 450 nm. The detection limit for glutamate and aspartate was about 1pmol (Herrera-Marschitz et al, 1996).

3.7.2 Pyruvate and Lactate analysis.

Pyruvate and lactate were separated on a Brownlee ion exchange reversed phase column (250 x 4,6 mm inner diameter, polypore H 10 μm) (Applied Biosystems Inc., San Jose, CA, USA). Volume of sample injected was 4 μl and 8 μl for brain and subcutaneous analysis respectively. The mobile phase used was 2.5 mM sulfuric acid at a flow rate of 0.3 ml/min maintained by a LKB 2150 LC pump (LKB, Bromma Sweden). A LKB 2151 variable wavelength monitor (LKB, Bromma, Sweden), at a 0.0025 absorbance range, and 214 nm wavelength was used for detection. The limit of detection was about 10 pmol (Ungerstedt and Hallström, 1987).

3.7.3 Monoamine analysis.

Dopamine and its acidic metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as 5-hydroxyindoleacetic acid (5-HIAA), the metabolite of 5-HT, were separated by a Biophase ODS 5 μm column (Diameter 250 x 4.6mm) and determined by HPLC coupled to an EC detector system (BAS Inc., West Lafayette, IN, USA). The equipm-ent comprises a refrigerated sample injector (CMA 200, CMA/Microdialysis AB) and a Spectra physics (San Jose, CA, USA) integrator. Volume injected was 5 μl. The elution was carried out with a phosphate buffer (0.1M Sodium salt of 1-octane sulfonic acid, 0.12M $NaH_2PO_4.H_2O$, 0.03% EDTA and 15% methanol, pH adjusted to 4.26, and made up to volume with distilled water) at a flow rate of 0.6 ml/min maintained with an HPLC pump (Jasco 880 pu, Japan). The limit of detection was about 5 fmol and 50 fmol for dopamine and its metabolites respectively (Herrera-Marschitz et al., 1996).

3.*8*. Preparation of the plant material

3.8.1 Collection of the plants

Clerodendron myricoides L. was collected from Debrezeit around Crater lake, 40 km East of Addis Ababa. *Cardiospermium helicacabum L.* was collected from Golede around Lake Elen, 5.2 km Northeast of Alem Tena town. *Heliotropium longifolium* was collected from the outskirts of Koka town on the road to Awassa, and *Teclea nobilis D.* was collected from a bush located 12 km Southeast of Shashemene on the road to Goba. All the plants were identified and a voucher Specimen was deposited in the National Herbarium, Addis Ababa University. The plant material after collection was shade dried, garbled to remove the part not required, and powdered using a grinding machine.

3.8.2 Extraction of the plant material

a) *Teclea nobilis-* the powdered leaf was successively macerated with petrol and 50% ethanol for about 24 hours. The ethanol extract was filtered and evaporated in vacuo to remove the alcohol. The remaining aqueous solution was freeze dried, weighted and reconstituted with distilled water to the required strength.

b) *Clerodendron myricoides-* the powdered root was successively macerated with petrol, 50% ethanol, and distilled water for about 24 hrs. The petrol extract was filtered and evaporated in vacuo. The residue was taken as extract 1. The hydroalcholic extract was also filtered and evaporated in vacuo to get extract 2. In both cases DMSO was used for reconstitution. The water extract was filtered and freeze dried and weighted, extract 3. And this was reconstituted with distilled water.

c) *Heliotropium longifolium*- the powdered whole plant was successively macerated with distilled water and absolute ethanol for about 24 hours. The water extract was filtered, freeze dried and weighted, extract 1. Extract 1 was reconstituted with distilled water to the required strength. The alcoholic extract was filtered and evaporated in vacuo. The residue was then dissolved in hot water and extracted with dichloromethane. The dichloromethane was evaporated in vacuo leaving a residue considered as extract 2. The aqueous layer was taken and extracted with ethyl acetate and the ethyl acetate was also evaporated in vacuo to get extract 3. DMSO was used to reconstitute extract 2 and 3 to the required strength.

d) *Cardiospermium helicacabum*- the powdered leaf was soaked in distilled water for about 24 hours. The mixture was then filtered and the filtrate (8%) was used as a bathing fluid.

3.9. Pharmacological treatment.

The various plant extracts were administered subcutaneously in different doses either three times with one hour interval between doses for *Clerodendron myricoides* and *Heliotropium longifolium*, or three times every hour, as well as twice daily for two days for *Teclea nobilis*. *Cardiospermium helicacabum* was used in the form of bathing fluid (8%). Brefiely, 400 ml of the extract was kept in a baker and this was in turn placed in a water bath adjusted to a suitable temperature so that the temperature of the extract would be 37°C. Then, the uterus horn was placed in the extract. Since asphyxial induction under hypothermic condition was carried out by reducing the temperature of the water bath and maintaining the temperature by adding ice, bathing with extracts was taken as an alternative.

3.10. Statistical analysis.

Values are expressed as percentage of each litter to describe survival rate, gasping, and vocalization; and as means ± SEM to describe respiratory frequency, spontaneous movement, and the level of the assayed substances as the concentration in the perfusates. The data were analyzed by F-ANOVA followed by student t-test for unequal number of samples. A level of $P< 0.05$ for a two tailed test was considered critical for statistical significance. Depending on the experimental condition grouping was done as follows; for short-term effect study, control (spontaneous and cesarean delivered pups), mild asphyxia (< 11 min), moderate asphyxia (< 20 min), and severe asphyxia (20 min and above). For therapeutic effect investigation; control (saline injected), treatment (extract treated).

4. RESULTS

4.1. Behavioral observation.

As shown in Table 1, a progressive decrease in the rate of survival was observed with the length of asphyxia. At 37°C, the normal body temperature, 100% survival was observed up to a 16 min asphyxia period; while about 40%, 28%, and 17% survival was observed after 19-20 min, 20-21 min, and 21-22 min asphyxia periods respectively. Asphyxia exposure longer than 22 min was inevitably associated with 100% mortality. During the early postnatal period (0-80 min), the effect of asphyxia was evident on the breathing efficacy, motility, color of the skin, and vocalization. Spontaneous and cesarean delivered pups started regular breathing almost immediately after delivery was completed, and showed a pink colored skin, intensive vocalization and motility. In contrast, asphyxic pups recovered slowly, and in pups exposed to moderate and severe asphyxia; gasping, cyanosis, tremor, sporadic clonic movement or no movement at all, and absence of vocalization were observed, even 80 min after delivery. The survival pattern of the pups after given to surrogate mothers was 97% (control), 97% (mild asphyxia), 86% (moderate asphyxia), and 82% (severe asphyxia), as assessed 1-8 days after delivery. Following 80 min-8 days after delivery, surviving asphyxic pups increased in body weight (Table 2) in a manner similar to control pups. No significant difference was observed in the increase in body weight, however, some neurological symptoms, such as slight tremor and rigidity affecting the hind legs, could be assessed in pups exposed to severe asphyxia at postnatal days 4 and 8. Since no significant difference was observed between spontaneous and cesarean delivered pups in any of the measured parameters, all comparisons were made against the cesarean delivered pups.

Table 1. Short-term effects of perinatal asphyxia at 37°C, monitored by direct observation in 40-80 min following delivery.

Experimental condition	Body weight (g)	Survival	Respiratory frequency	Gasping	Vocalization	Color of the skin	Spontaneous movement
Spontaneous delivery, (n=35)	5.87±0.12	100%	64±1	0%	100%	Pink	3.89±0.05
Cesarean delivery, (n=62)	5.63±0.09	100%	66±1	0%	100%	Pink	3.9±0.04
Asphyxia							
2-3 min (n=55)	5.61±0.09	100%	64±1	0%	100%	Pink	3.74±0.06
5-6 min (n=53)	5.5 ± 0.09	100%	63 ± 2	0%	100%	Pink	3.55 ± 0.07
10-11 min (n=58)	5.49 ± 0.09	100%	60 ± 2	0%	100%	Pink	3.34 ± 0.08
15-16 min (n=58)	5.63 ± 0.09	100%	52 ± 2	10%	73%	Pink/Pale	2.48 ± 0.13
19-20 min (n=128)	5.71 ± 0.08	40%	32 ± 2	47%	2%	Pale	0.39 ± 0.08
20-21 min (n=290)	5.51 ± 0.08	28%	28 ± 2	48%	0%	Pale	0
21-22 min (n=180)	5.67 ± 0.07	17%	22 ± 2	84%	0%	Pale	0
22-23 min (n=16)	5.51 ± 0.17	0%	-	-	-	Pale	-

Table 2. Body weight of asphyxic pups following 80 min-8 days after delivery.

Age	80 min,	24 hrs.	48 hrs.	4 days	8 days
Spont.,(N=8-21)	5.51± 0.18	6.65± 0.27	7.75± 0.33	11.79±0.39	14.2 ± 0.62
Ces., (N= 7-13)	5.39± 0.23	6.11± 0.26	7.30± 0.17	9.58 ± 0.42	16.81±0.94
2-3 min, (N=8-12)	5.32± 0.21	6.41± 0.27	7.48± 0.32	9.73 ± 0.35	16.9 ± 1.02
5-6 min, (N=8-12)	5.52± 0.26	6.53± 0.22	7.38± 0.32	8.88 ± 0.31	16.34 ± 0.5
10-11, (N=9-14)	5.29± 0.19	6.42 ± 0.4	7.22± 0.24	8.85 ± 0.41	16.31 ± 0.7
15-16, (N=9-14)	5.89± 0.17	6.18± 0.29	6.97± 0.24	8.79 ± 0.81	16.37 ± 0.8
19-20, (N=6-14)	5.47± 0.12	6.00± 0.26	6.48± 0.36	9.59 ± 0.3	16.58±0.77
20-21, (N=6-12)	5.18± 0.17	5.33± 0.34	6.47± 0.26	10.05±0.47	16.21±0.71
21-22, (N=5-10)	5.31± 0.14	4.98± 0.22	5.96± 0.31	9.04 ± 0.59	16.23±2.21

4.2. pH measurement

The pH of the brain and heart varied with the duration of asphyxia (Fig. 1). Brain pH was about 7.3 in spontaneous and cesarean delivered controls, and decreased with the length of asphyxia. A significant decrease ($P < 0.01$) was first observed following 10-11 min of asphyxia (7.18±0.01,N=6), and a maximum following 21-22 min of asphyxia (6.18±0.02, N=6). Heart pH was about 7.35 in spontaneous and cesarean delivered controls and decreased with the length of asphyxia. A significant decrease ($P < 0.05$) in heart pH was first observed following 5-6 min asphyxia, with a maximum following 21-22 min asphyxia (7.01±0.02; N=6).

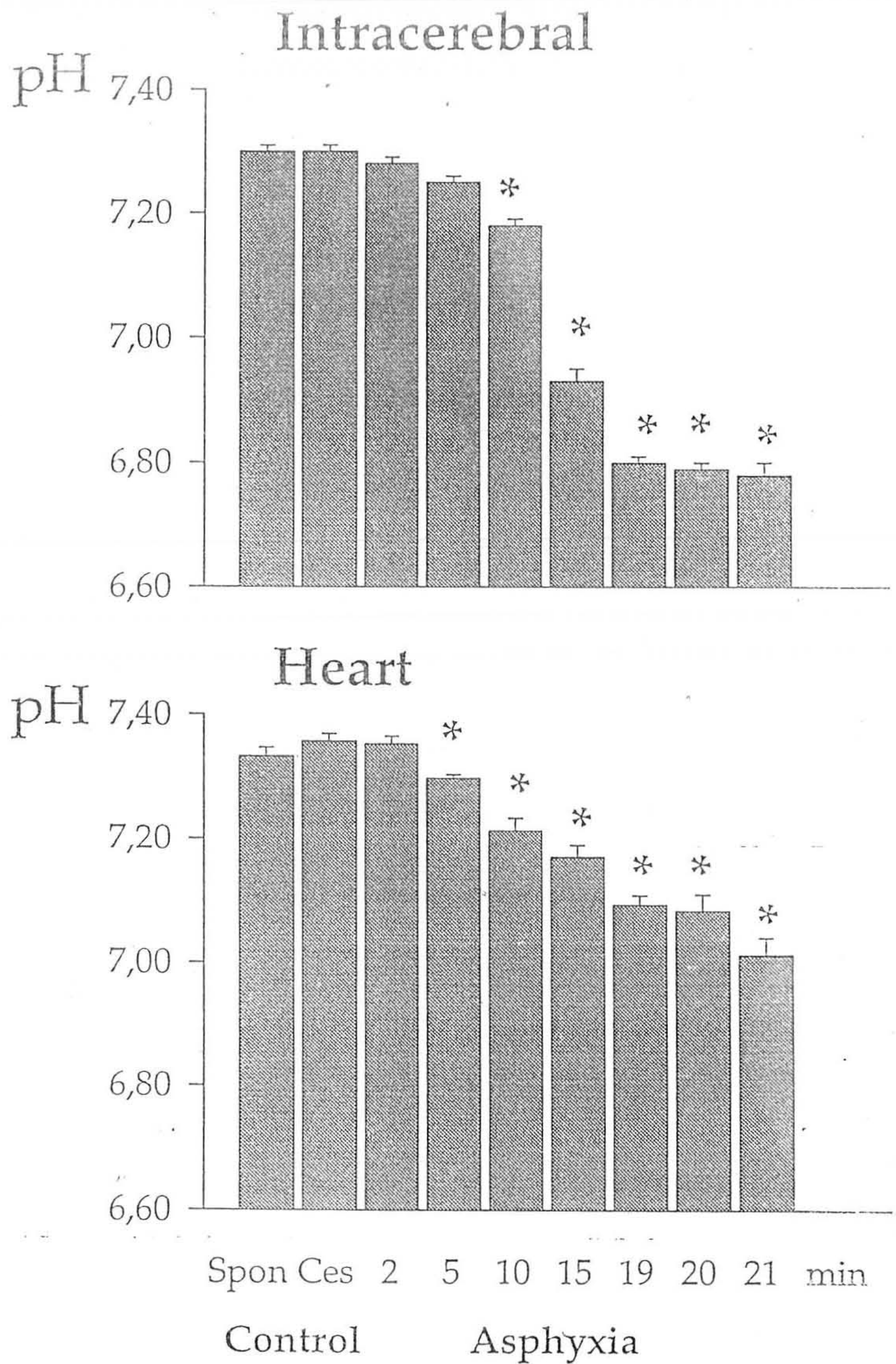

Fig. 1 pH measured from the brain (top) and heart (bottom) 40 min after delivery of rat pups. P< 0.05 vs. controls, N=6.

4.3. In vitro recovery.

The probes showed approximately 15.3%, 20.1%, 20.5%, and 25.7% in vitro recovery for aspartate, glutamate, pyruvate, and lactate, respectively.

4.4. Biochemical analysis.

4.4.1 Extracellular levels monitored in subcutaneous tissue

The levels of pyruvate, lactate, glutamate, and aspartate analyzed from subcutaneous tissue of 80 min old spontaneous and cesarean delivered control pups and pups exposed to asphyxia (2-22 min) are shown in Table 3. Pyruvate level measured 80 min after delivery was significantly increased by mild asphyxia. A maximum (2-3 fold) was observed following 5-6 min of asphyxia. After asphyxic period longer than 6 min, pyruvate level started to decrease, however, the level was still significantly higher than that of the control ($P< 0.01$).

Table 3. Levels of pyruvate, lactate, glutamate, and aspartae in μM measured from subcutaneous tissue of rat pups following 80 min after delivery. $P< 0.05$ vs. controls.

Duration of asphyxia	Pyruvate	Lactate	Glutamate	Aspartate
Spontaneous (N=14)	25.38 ± 1.51	758.94 ± 61.8	23.46 ± 4.00	1.92 ± 0.21
Cesarean (N=13)	26.32 ± 3.56	769.33 ± 42.8	24.36 ± 3.14	1.9 ± 0.24
2-3 min (N=12)	54.6 ± 5.65*	1178.6 ± 128*	38.59 ± 6.92*	3.45 ± 0.59*
5-6 min (N=12)	62.27 ± 10.03*	1575.86 ± 153*	37.57 ± 5.6*	3.49 ± 0.53*
10-11 min (N=14)	48.13 ± 5.4*	1583.26 ± 116*	36.41 ± 4.23*	2.72 ± 0.34*
15-16 min (N=13)	46.92 ± 3.1*	1756.62 ± 158*	35.53 ± 4.33*	2.99 ± 0.34*
19-20 min (N=14)	40.58 ± 5.2*	2484.60 ± 318*	27.55 ± 1.26	2.54 ± 0.4*
20-21 min (N=12)	38.64 ± 1.63*	2669.74 ± 357*	27.29 ± 3.1	2.04 ± 0.21
21-22 min (N=10)	33.92 ± 3.3	1546.93 ± 165*	20.73 ± 3.0	1.67 ±0.3

The level of pyruvate observed after 21 min was close to that of the control. Lactate level increased (2-4 fold) in pups exposed to asphyxia ($P < 0.01$, in all cases) a maximum increase being at 20-21 min. A decline in lactate level was also observed after 21 min compared to the maximum. The levels of glutamate and aspartate was also increased (about two fold) until 16 min of asphyxia. After asphyxic period longer than 16 min the levels declined and reached the control levels. Monoamines were not detected in subcutaneous tissue.

Twenty four hrs after delivery, the subcutaneous levels of all the analyzed substances decreased approximately by 1.5-4 fold in asphyxiated pups as compared to the newborn asphyxiated pups. In contrast, in control pups the level of pyruvate was almost similar while the levels of lactate (Table 4), glutamate and aspartate (Table 5) decreased by 10-18% compared to the newborn controls. With increasing age, a slight increase was observed in the level of pyruvate, whereas lactate, glutamate, and aspartate levels showed a decreasing pattern in control pups. In asphyxiated pups, the levels of pyruvate, lactate, glutamate, and aspartate decreased following postnatal day 2 and tend to approach that of the control with age.

Table 4. Levels of extracellular pyruvate and lactate measured in μM from subcutaneous tissue of rat pups following 24 hrs, 48 hrs, 4 days, and 8 days after delivery.

Age	24 hrs		48 hrs		4 days		8 days	
Duration of asphyxia	Pyruvate	Lactate	Pyruvate	Lactate	Pyruvate	Lactate	Pyruvate	Lactate
Spont. (N= 8-18)	27.81 ± 2.95	625.4 ± 32	28.74 ± 2.13	256.4 ± 21	30.47 ± 3.06	217.3 ± 23	32.25 ± 3.57	303.5 ± 42
Ces. (N=7-14)	28.81 ± 3.84	620.8 ± 77	31.09 ± 4.22	311.3 ± 32	31.15 ± 3.15	260.2 ± 25	32.92 ± 2.96	287.9 ± 29
2-3 min (N=8-12)	23.73±2.49	634 ±11.2	22.96 ± 4.53	427.2 ± 54.9	21.56 ± 4.00	317.6 ± 45.5	24.47 ± 5.00	272 ± 48
5-6 min (N=8-12)	31.38 ± 3.91	616.3 ± 56.3	19.45 ± 5.5	332.5 ± 67	20.81 ± 4.41	328.7 ± 64.5	24.57 ± 2.55	305.5 ± 47.2
10-11 min(N=9-12)	37.13 ± 8.6	655.4 ± 48	18.66 ± 2.27	317.7 ± 30	16.63 ± 2.14	305.2 ± 37	22.39 ± 3.24	319 ± 31
15-16 min (N=7-11)	32.40 ± 5.38	585.1 ± 120	29.78 ± 6.17	502 ± 60	27.39 ± 4.76	499.6 ± 87	23.22 ± 2.68	258.2 ± 48.6
19-20 min (N=6-10)	30.24 ± 4.4	608.2 ± 90	23.53 ± 3.57	502 ± 92	19.07 ± 2.97	406.7 ± 93	21.6 ± 3.3	258.2 ± 49
20-21 min (N=6-8)	33.4 ± 7.76	646.3 ± 171	22.15 ± 1.41	586.2 ± 50.6	21.01 ± 1.93	266.3 ± 53.1	22.67 ± 5.18	278.9 ± 34.7
21-22 min (N=5-7)	31.6 ± 3.96	507.6 ± 78.5	28.45 ± 1.41	462.3 ± 74.7	21.54 ± 3.09	331.4 ± 49.8	22.99 ± 1.21	293.9 ± 56.7

Table 5. Levels of extracellular glutamate and aspartate measured in μM from subcutaneous tissue of rat pups following 24 hrs, 48 hrs, 4 days, and 8 days after delivery.

Age	24 hrs		48 hrs		4 days		8 days	
Duration of asphyxia	Glutamate	Aspartate	Glutamate	Aspartate	Glutamate	Aspartate	Glutamate	Aspartate
Spont. (N= 8-18)	18.12 ± 2.18	1.71 ± 0,21	9.16 ± 1.39	1.23 ± 0.15	10.9 ± 1.9	1.14 ± 0.18	10.27 ± 2.62	1.66 ± 0.28
Ces. (N=7-14)	19.75 ± 3.28	1.92 ± 0.31	15.30 ± 2.62	1.79 ± 0.25	10.75 ± 1.89	1.39 ± 0.18	10.27 ± 2.3	1.59 ± 0.39
2-3 min (N=8-12)	21.82 ± 3.08	1.79 ± 0.29	20.9 ± 4.09	1.74 ± 0.33	18.62 ± 4.86	1.53 ± 0.37	10.49 ± 3.22	1.41 ± 0.19
5-6 min (N=8-12)	24.19 ± 4.39	1.91 ± 0.4	18.23 ± 4.2	1.85 ± 0.15	17.36 ± 4.71	1.69 ± 0.67	14.65 ± 3.58	1.56 ± 0.26
10-11 min(N=9-12)	20.98 ± 3.41	1.73 ± 0.34	17.43 ± 3.44	1.9 ± 0.22	15.05 ± 3.59	1.58 ± 0.23	13.66 ± 2.6	1.59 ± 0.25
15-16 min (N=7-11)	16.21 ± 2.97	1.59 ± 0.42	17.4 ± 0.44	1.68 ± 0.38	17.1 ± 5.33	1.67 ± 0.22	13.69 ± 3.82	1.79 ± 0.31
19-20 min (N=6-10)	17.22 ± 4.73	1.99 ± 0.41	16.7 ± 4.34	1.64 ± 0.17	15.63 ± 1.61	1.77 ± 0.31	8.75 ± 0.94	1.46 ± 0.13
20-21 min (N=6-8)	20.04 ± 6.89	1.51 ± 0.29	22.6 ± 4.55	1.73 ± 0.1	17.67 ± 3.81	1.52 ± 0.32	10.5 ± 1.4	1.32 ± 0.38
21-22 min (N=5-7)	20.77 ± 2.62	2.28 ± 0.36	20.45 ± 2.68	1.45 ± 2.68	14.8 ± 4.19	1.46 ± 0.22	9.68 ± 2.27	1.43 ± 0.27

4.4.2 Tissue levels monitored in striatum.

The levels of different compounds analyzed in tissue samples taken from striatum of 80 min old spontaneous and cesarean delivered control pups and pups subjected to different periods of asphyxia are shown in Table 6. Approximately 80 min after delivery, striatal pyruvate level was increased until 11 min of asphyxia ($P < 0.001$), with a maximum increase at 10-11 min (about two fold). After 11 min, it started to decline and went back to a level which was not significantly different to that of the control. Asphyxic periods up to 20 min were associated with an increase in lactate level, with a maximum increase (five fold) noted at 19-20 min of asphyxia. A significant increase ($P < 0.001$) was observed in lactate level for all asphyxic periods longer than 10 min. The level, however, started to decline after a period of 20 min. The concentration of glutamate and aspartate was also increased up to 11 min of asphyxia, with a maximum increase (2-3 fold) at 10-11 min. Thereafter, it went below control level. It was noted that mild asphyxia increased the levels of monoamines as compared to the controls (Table 6). A maximum increase was observed at 10-11 min with dopamine (2-3 fold), DOPAC (two fold), HVA (1-2 fold), 5-HIAA (2-3 fold). After 16 min, the levels tend to return to control level .

Table 6. Levels of pyruvate, lactate, glutamate, aspartate, in μmol/gm and monoamines in nmol/gm measured from tissue samples taken from striatum of rat pups following 80 min after delivery. P< 0.05 vs. controls.

Time of asphyxia	Pyruvate	Lactate	Glutamate	Aspartate	Dopamine	DOPAC	HVA	5-HIAA
Spont. (N= 7)	2.14 ± 0.24	3.23 ± 0.41	2.7 ± 0.7	0.82 ±0.2	3.0 ± 0.4	0.34 ± 0.09	0.61 ± 0.07	3.48 ± 0.41
Cesarean(N= 6)	2.48 ± 0.35	3.88 ± 0.52	2.55 ± 0.35	0.8 ± 0.07	3.51 ± 0.42	0.36 ± 0.03	0.62 ± 0.08	3.58 ± 0.4
2-3 min (N= 8)	3.18 ± 0.28*	5.48 ± 1.07*	4.82 ± 0.7*	1.25 ± 0.2*	5.55 ± 0.5*	0.38 ± 0.06	0.53 ± 0.05	5.25 ± 0.9*
5-6 min (N= 5)	4.81 ± 0.26*	6.96 ± 1.32*	3.57 ± 0.7*	1.33 ± 0.2*	6.17 ± 1.3*	0.51 ± 0.1*	0.58 ± 0.07	6.25 ± 0.8*
10-11min (N= 5)	5.74 ± 0.75*	11.61 ± 1.0*	6.4 ± 0.4*	1.55 ± 0.2*	7.94 ± 2.1*	0.62 ± 0.2*	0.91 ± 0.2*	6.29 ± 0.5*
15-16 min (N= 5)	2.67 ± 1.1	16.06 ± 2.3*	2.58 ± 0.34	0.38 ± 0.04	5.49 ± 1.2*	0.56 ± 0.1*	0.67 ± 0.11	4.68 ± 1.0*
19-20 min (N= 4)	1.87 ± 0.19	16.68 ± 1.9*	2.52 ± 0.69	0.31 ± 0.1	2.83 ± 0.92	0.31 ± 0.04	0.43 ± 0.12	4.09 ± 0.52
20-21 min (N=5)	1.95 ± 0.22	13.05 ± 1.8*	2.39 ± 0.28	0.32 ± 0.03	3.18 ± 0.52	0.31 ± 0.1	0.72 ± 0.08	4.29 ± 0.67
21-22 min(N= 5)	1.52 ± 0.24	11.24 ± 1.6*	2.35 ± 0.35	0.32 ± 0.06	2.69 ± 0.48	0.27 ± 0.06	0.53 ± 0.04	3.81 ± 0.51

Twenty four hours after delivery, there was a reduction in the level of pyruvate about 2-4 fold, in all asphyxiated pups compared to the newborn asphyxiated ones (Table 7). The levels of glutamate and aspartate (Table 8) were slightly decreased in those pups exposed to mild asphyxia (particularly those exposed to <10 min) whilst a slight increase in the levels of glutamate and aspartate were observed in those pups exposed to moderate and severe asphyxia. A significant decrease ($P<0.01$) in the levels of aspartate, glutamate, and pyruvate were observed at 10-11 min of asphyxia. Lactate level (Table 7) showed an increase (2-3 fold) in <6 min asphyxic pups, however, the level was not greatly affected in pups exposed to >6 min asphyxia. In control pups, the level of pyruvate decreased whereas that of lactate increased (about three fold in both cases) compared to the newborn controls. No difference, however, was observed in the levels of glutamate and aspartate.

In control pups with increasing age (Postnatal day 2-8), the levels of pyruvate, glutamate, and aspartate were almost similar to that of the 24 hrs levels, whereas in case of lactate the level was almost the same until postnatal day 4 and decreased significantly ($P<0.05$) at postnatal day 8 (Table 7 & 8). In asphyxic pups, the levels of pyruvate, glutamate, and, aspartate decreased and approached that of the control with age, however, a significant drop ($P<0.05$) in lactate was observed following postnatal day 4.

Following 24 hrs, the levels of monoamines such as HVA (Table 10), and 5-HIAA decreased significantly ($P< 0.01$) (2-4 fold) in pups exposed to moderate and severe asphyxia. The decrease was not significant in control pups and pups exposed to mild asphyxia. Nevertheless, in pups exposed to mild asphyxia the decrease in HIAA was still significant ($P< 0,05$). The level of DOPAC (Table 9) increased in both control and asphyxic pups, and the level of dopamine (Table 9) slightly increased in control and severely asphyxiated pups but decreased in pups exposed to mild and moderate asphyxia.

Table 7. Levels of pyruvate and lactate in µmol/gm measured from tissue samples taken from striatum of rat pups following 24 hrs, 48 hrs, 4 days, and 8 days after delivery.

Age	24 hrs		48 hrs		4 days		8 days	
Duration of asphyxia	Pyruvate	Lactate	Pyruvate	Lactate	Pyruvate	Lactate	Pyruvate	Lactate
Spont. (N= 5-6)	0.86 ± 0.13	8.40 ± 0.3	0.46 ± 0.04	8.33 ± 0.67	0.55 ± 0.03	9.05 ± 0.86	0.61 ± 0.03	3.83 ± 0.57
Cesarean (N= 4-6)	0.61 ± 0.03	8.17 ± 0.87	0.45 ± 0.07	7.71 ± 0.96	0.51 ± 0.07	7.07 ± 1.1	0.61 ± 0.0.03	3.93 ± 0.18
2-3 min (N= 5-8)	0.75 ± 0.09	9.13 ± 0.72	0.5 ± 0.03	10.12 ± 0.88	0.56 ± 0.08	4.05 ± 0.51	0.60 ± 0.05	5.51 ± 1.1
5-6 min (N= 5)	0.82 ± 0.08	9.98 ± 1.09	0.31 ± 0.05	10.58 ± 2.11	0.53 ± 0.03	3.55 ± 0.28	0.52 ± 0.05	3.64 ± 0.66
10-11 min (N= 5)	0.55 ± 0.05	12.34 ± 1.15	0.38 ± 0.02	8.98 ± 1.52	0.47 ± 0.04	4.21 ± 0.61	0.53 ± 0.02	4.14 ± 0.4
15-16 min (N= 3-5)	0.79 ± 0.18	11.14 ± 1.94	0.49 ± 0.04	11.87 ± 0.51	0.6 ± 0.09	5.68 ± 1.18	0.66 ± 0.06	3.7 ± 0.42
19-20 min (N= 4-5)	0.44 ± 0.07	14.69 ± 1.26	0.56 ± 0.01	13.34 ± 1.58	0.55 ± 0.04	4.44 ± 0.31	0.48 ± 0.0.02	3.19 ± 0.39
20-21 min (N= 4-5)	0.64 ± 0.09	11.20 ± 1.35	0.52 ± 0.04	11.08 ± 1.33	0.56 ± 0.07	6.03 ± 0.47	0.61 ± 0.02	2.98 ± 0.08
21-22 min (N= 5)	0.67 ± 0.04	10.74 ± 1.96	0.4 ± 0.06	5.03 ± 0.56	0.78 ± 0.03	5.88 ± 0.52	0.45 ± 0.03	5.4 ± 0.83

Table 8. Levels of glutamate and aspartate in µmol/gm measured from tissue samples taken from striatum of rat pups following 24 hrs, 48 hrs, 4 days, and 8 days after delivery.

Age	24 hrs		48 hrs		4 days		8 days	
Duration of asphyxia	Glutamate	Aspartate	Glutamate	Aspartate	Glutamate	Aspartate	Glutamate	Aspartate
Spont. (N= 5-6)	3.83 ± 0.26	0.79 ± 0.07	3.2 ± 0.5	0.81 ± 0.08	3.98 ± 0.71	0.85 ± 0.14	2.49 ± 0.29	0.46 ± 0.05
Cesarean (N= 4-6)	2.26 ± 0.18	0.68 ± 0.06	3.54 ± 0.61	0.77 ± 0.01	2.58 ± 0.27	0.68 ± 0.07	2.68 ± 0.17	0.5 ± 0.02
2-3 min (N= 5-8)	3.15 ± 0.15	0.8 ± 0.18	3.75 ± 0.43	0.72 ± 0.02	2.06 ± 0.33	0.72 ± 0.16	2.27 ± 0.22	0.47 ± 0.06
5-6 min (N= 5)	3.53 ± 0.45	0.88 ± 0.15	2.54 ± 0.29	0.87 ± 0.11	2.19 ± 0.21	0.65 ± 0.08	2.86 ± 0.22	0.6 ± 0.09
10-11 min (N= 5)	2.51 ± 0.29	0.61 ± 0.08	2.54 ± 0.29	0.68 ± 0.02	2.24 ± 0.4	0.59 ± 0.1	2.48 ± 0.25	0.44 ± 0.02
15-16 min (N= 3-5)	2.95 ± 0.19	0.62 ± 0.08	2.37 ± 0.18	0.71 ± 0.01	2.39 ± 0.43	0.5 ± 0.14	2.53 ± 0.14	0.55 ± 0.05
19-20 min (N= 4-5)	2.79 ± 0.11	0.57 ± 0.08	2.33 ± 0.24	0.63 ± 0.03	2.53 ± 0.39	0.57 ± 0.09	2.11 ± 0.23	0.41± 0.04
20-21 min (N= 4-5)	2.7 ± 0.24	0.85 ± 0.1	2.33 ± 0.22	0.77 ± 0.07	2.29 ± 0.37	0.52 ± 0.12	1.85 ± 0.21	0.61 ± 0.04
21-22 min (N= 5)	2.37 ± 0.22	0.78 ± 0.14	3.3 ± 0.29	0.75 ± 0.07	2.74 ± 0.41	0.66 ± 0.13	1.78 ± 0.14	0.34 ± 0.03

Table 9. Levels of dopamine and DOPAC in nmol/gm measured from tissue samples taken from striatum of rat pups following 24 hrs, 48 hrs, 4 days, and 8 days after delivery.

Age	24 hrs		48 hrs		4 days		8 days	
Duration of asphyxia	Dopamine	DOPAC	Dopamine	DOPAC	Dopamine	DOPAC	Dopamine	DOPAC
Spont. (N= 5-6)	4.76 ± 0.5	3.41 ± 0.53	4.17 ± 0.63	6.18 ± 0.79	18.49 ± 1.62	6.73 ± 1.03	28.86 ± 4.24	6.86 ± 0.29
Cesarean (N= 4-6)	4.19 ± 0.33	3.11 ± 0.39	5.26 ± 1.63	5.69 ± 2.09	17.48 ± 0.44	6.87 ± 0.26	29.14 ± 2.39	6.61 ± 0.69
2-3 min (N= 5-8)	3.04 ± 0.32	3.63 ± 0.54	6.62 ± 1.31	2.45 ± 0.57	11.33 ± 0.35	1.74 ± 0.37	33.33 ± 4.02	3.27 ± 0.31
5-6 min (N= 5)	3.93 ± 0.69	3.93 ± 0.69	6.84 ± 1.87	3.24 ± 0.86	17.17 ± 2.16	2.12 ± 0.35	32.83 ± 2.35	2.69 ± 0.23
10-11 min (N= 5)	3.48 ± 0.84	1.51 ± 0.33	6.29 ± 1.76	1.27 ± 0.27	15.1 ± 1.79	1.46 ± 0.19	33.18 ± 2.96	2.89 ± 0.31
15-16 min (N= 3-5)	2.43 ± 0.63	2.35 ± 0.38	8.38 ± 1.47	1.31 ± 0.19	16.25 ± 0.53	2.38 ± 0.23	29.68 ± 1.05	2.38 ± 0.18
19-20 min (N= 4-5)	1.72 ± 0.24	2.41 ± 0.36	7.72 ± 1.07	2.65 ± 0.79	15.05 ± 1.63	2.54 ± 0.26	21.57 ± 1.38	2.79 ± 0.16
20-21 min (N= 4-5)	4.66 ± 0.65	1.62 ± 0.46	8.79 ± 1.46	2.78 ± 0.46	11.53 ± 1.27	2.41 ± 0.62	20.5 ± 1.41	2.71 ± 0.27
21-22 min (N= 5)	5.27 ± 0.89	1.67 ± 0.32	8.04 ± 1.13	2.55 ± 0.24	16.14 ± 1.01	2.03 ± 0.22	21.8 ± 2.52	2.21 ± 0.33

No significant difference in dopamine level was observed in control and severely asphyxiated pups following 24 hrs after delivery. As age increased, there was an increase in the levels of dopamine, DOPAC, HVA, and 5-HIAA in both control and asphyxic pups with less pronounced increase in the most asphyxic pups.

Table 10. Level of HVA in nmol/gm measured from tissue samples taken from striatum of rat pups following 24 hrs, 48 hrs, 4 days, and 8 days after delivery.

Duration of asphyxia	24 hrs.	48 hrs.	4 days	8 days
Sponta. (N= 5-6)	0.59 ± 0.13	0.5 ± 0.04	2.2 ± 0.47	3.76 ± 0.43
Cesarean (N=4-6)	0.33 ± 0.03	0.79 ± 0.22	1.5 ± 0.27	4.21 ± 0.32
2-3 min (N= 5-8)	0.37 ± 0.03	1.34 ± 0.24	1.57 ± 0.33	4.15 ± 0.37
5-6 min (N= 5)	0.38 ± 0.07	0.45 ± 0.07	1.57 ± 0.18	3.91 ± 0.37
10-11 min (N= 5)	0.34 ± 0.06	1.09 ± 0.16	1.38 ± 0.2	3.61 ± 0.4
15-16 min (N=3-5)	0.68 ± 0.16	1.07 ± 0.02	1.85 ± 0.39	3.57 ± 0.26
19-20 min (N=4-5)	0.27 ± 0.03	1.11 ± 0.28	1.66 ± 0.21	2.6 ± 0.22
20-21 min (N=4-5)	0.32 ± 0.03	1.33 ± 0.12	1.44 ± 0.23	2.38 ± 0.21
21-22 min (N= 5)	0.44 ± 0.06	1.21 ± 0.1	3.21 ± 0.38	2.11 ± 0.39

4.5 Role of hypothermia and plant extracts on survival.

4.5.1 Role of hypothermia

When asphyxia was induced under hypothermic condition; 30°C (moderate hypothermia) and 15°C (profound hypothermia) there was a significant increase ($P < 0.001$) in the survival rate compared to the survival pattern observed at 37°C (Fig. 2). With moderate hypothermia S_{100} (time at which survival is 100%) was 30 min whereas S_{100} was 100 min with profound hypothermia. S_{50} (time at which survival is 50%) was 43 min and 120 min respectively for moderate and profound hypothermia. In profound hypothermia, S_0 (time at which survival is

nill) was about three and seven times longer respectively, compared to moderate hypothermia and normothermia (Table 11).

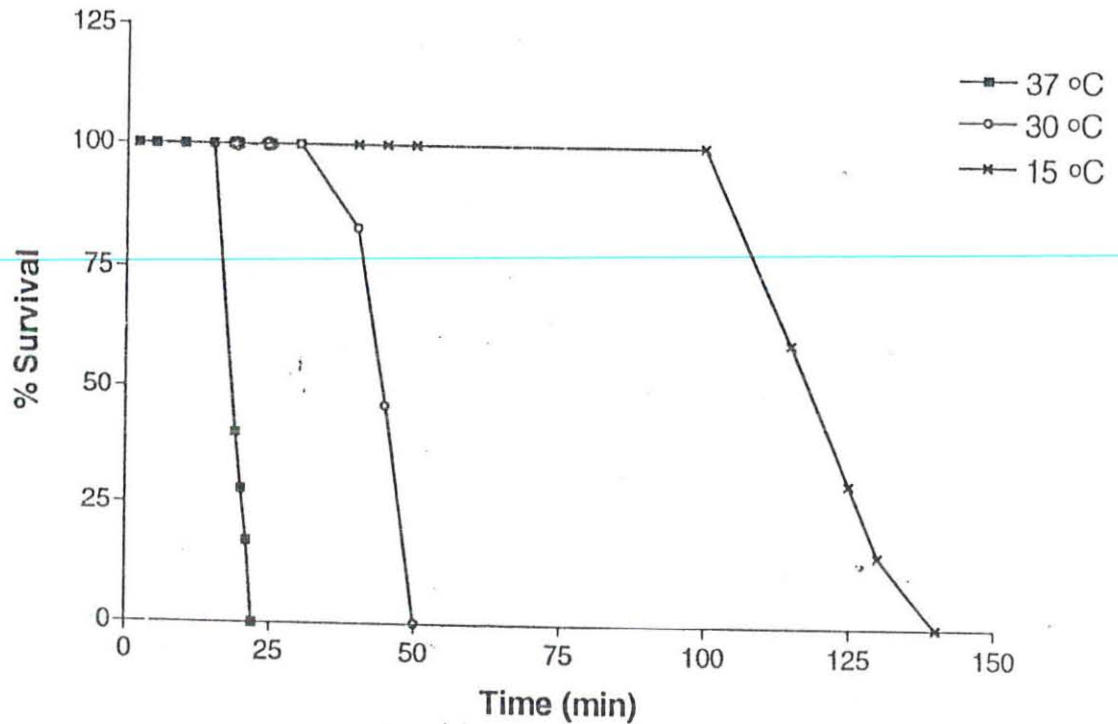

Fig. 2. Survival pattern of rat pups after induction of asphyxia at normothermia (37°C), moderate (30°C) and profound (15°C) hypothermia. P< 0,001 vs. controls, N= 5-10.

Table 11. Survival indices of rat pups at different temperature.

Survival index	37°C	30°C	15°C
S_{100}	15 min	30 min	100 min
S_{50}	17 min	43 min	120 min
S_0	22 min	50 min	140 min

S_{100}- time for 100% survival, S_{50}-time for 50% survival, S_0-time for 0% survival

4.5.2 Role of plant extracts

After treatment with plant extracts, there was no significant difference in the survival pattern of the pups compared to the saline injected control groups (Table 12). In both treated and control group S_{50} was between 16-17 min. The vehicle used to reconstitute the organic extracts was DMSO, and it did not affect the survival pattern of the rat pups. Amongst the plant extracts screened, the ethyl acetate and water extract of *Heliotropium longifolium* were able to cross the 22 min time limit, at normal body temperature, for survival. When the pups were taken out after 24 min, they gasped and tried to survive for sometime but, their effort did not last long. They died in the 40 min recovery time. The water extract of the roots of *Clerodendron myricoides* was toxic, it killed the mother after administration.

Table 12. Effect of plant extracts on survival pattern after induction of asphyxia at 37°C

Plant	Part used	Extract type	Dose	Route	Effect on survival						
					15-16	19-20	20-21	21-22	22-23	23-24	24-25
Control (n= 5-10)		Saline	2 ml	Sc	100%	35%	25%	15%	0%	0%	0%
T.nobilis (n= 5-8)	Leaf	Hydroalcholic	280mg/kg	Sc	100%	40%	25%	14%	0%	0%	0%
H. longifolium (n= 5-12)	Whole plant	Extract 1	325mg/kg	Sc	100%	42%	28%	28%	0%	0%	0%
		Extract 2	500mg/kg	Sc	100%	30%	12%	11%	0%	0%	0%
		Extract 3	400mg/kg	Sc	100%	33%	14%	10%	0%	0%	0%
C. Myricoides (n= 6-8)	Root	Extract 1	400mg/kg	Sc	100%	40%	18%	15%	0%	0%	0%
		Extract 2	400mg/kg	Sc	100%	37%	16%	13%	0%	0%	0%
C.helicacabum (n= 5-8)	Leaves	Water	8%	Bath	100%	33%	14%	12%	0%	0%	0%

Values are expressed as percent survival.

5. DISCUSSION

The rat model used in the present study was non-invasive and largely mimicked the conditions resulting in asphyxia during human labor. The model allows to study both short and long-term metabolic and neuropathological changes after an asphyxial insult.

5.1 Behavioral observation

The rate of survival was dependent upon the duration of asphyxia and the temperature. At 37°C, the normal body temperature, the rate of survival rapidly decreased following exposure to perinatal asphyxia longer than 16 min and eventually came to 0% when asphyxia was longer than 22 min. Using several vegetative parameters related to respiratory and cardiovascular function, it was demonstrated that pups surviving prolonged asphyxia showed persistent deficit in respiration and peripheral circulation reflected as a change in skin color. Thus, a general cardio-respiratory failure appears to occur that may result in brain damage. The above changes were also accompanied by a decrease in motility and vocalization, indicating a general compromise of the central nervous system. The above mentioned changes could be attributed to a progressive and prolonged decrease in tissue oxygen brought about by perinatal asphyxia. Moreover large proportion of pups exposed to moderate (14%) and severe (18%) asphyxia died after given to surrogate mothers as compared to control (3%) and mild (3%) asphyxic pups. The death of control and mild asphyxic pups may be explained by surrogate mother rejection whereas the death of moderately and severely asphyxiated pups perhaps be caused by both rejection and asphyxia. Thus, this probably shows that moderate and severe perinatal asphyxia can be related to death even during early nursery period.

During the postnatal period, pups surviving perinatal asphyxia and accepted by surrogate mothers, normally developed along with control rats. Moreover, it was found here

that asphyxic pups (which were normally accepted by surrogate mothers) did not differ from controls in body weight indicating a normal maturation and physical development. The neurological symptoms assessed in very asphyxic pups is in agreement with biochemical and behavioral changes observed four weeks (Bjelke et al., 1991) and 6-12 months (Herrera-Marschitz et al., 1994; Loidl et al., 1994) following perinatal asphyxia. Hence, alterations in some CNS functions appear to occur immediately after perinatal asphyxia as well as later in development.

5.2 pH determination

Asphyxia can lead to cellular acidosis via sources of H^+ ions in addition to lactic acid. Major sources include hydrolysis of ATP, NADH + H^+ accumulated during cellular oxygen debt, and as end product of anaerobic glycolysis. The decrease in brain and heart pH appears to be directly correlated with the length of asphyxia. Change in pH was first observed in the heart following 5-6 min asphyxia, compared to the brain. Although the heart seems sensitive to short interruption of blood-oxygen supply initially, the decrease in pH in the heart was less extreme when asphyxia was prolonged indicating that the heart is probably better buffered than the brain under sustained asphyxia, that secures the survival of the pups even with possible ensuing brain damage. Oxygen deprivation along with a drop in pH may lead to persistent impairment in metabolism causing delayed neuronal death in brain regions that are sensitive to anoxic or ischemic damage. It has been observed that delayed neuronal death in striatum and other brain regions is most prominent following asphyxic periods longer than 19 min (Dell' Anna et al., 1996). In the present study it was demonstrated that the pH of these very pups was below 7.0, which is in agreement with the report that, pH 7.0 is the cutoff point for development of significant neuronal loss (Low, 1993) and with the opinion that, the more depressed the pH level, the more probable the development of brain injury (Myers, 1972).

5.3 Biochemical analysis

The ultimate outcome of perinatal asphyxial episode is either death or survival. Nevertheless, surviving pups may develop neurological deficits which may be associated to neurodegenerative disorders with an onset at late adult stage (Herrera-Marschitz, 1993; Dell' Anna et al., 1995). The cause of these deficits might be biochemical changes that occurred during the insult or later at the reoxygenation stage. Monitoring of these changes has given interesting results that supports their cause and effect relationship.

5.3.1 Changes observed in the levels of pyruvate and lactate after 80 min

Pyruvate is the major product of glycolysis which under aerobic conditions enters the mitochondria where it is completely metabolized to carbondioxide and water. Therefore, extracellular pyruvate level should increase whenever there is an increase in energy demand. If the supply of oxygen is insufficient, as in the case of asphyxia, further metabolism of pyruvic acid by the pyruvate dehydrogenase complex is inhibited by a high NADH + H^+:NAD ratio, which also promotes conversion of pyruvate to lactate, hence the accumulation of lactate is an indication of a shift from aerobic to anaerobic metabolism. Thus, the elevation observed in pyruvate level following mild asphyxia, 80 min after delivery may indicate an increased glycolysis, probably via increased activity of phosphofructokinase and pyruvate kinase (Siesjö, 1978; Lutz, 1992). Although stimulation of glycolysis can supplement oxidative phosphorylation under conditions of partial oxygen debt, it can never substitute mitochondrial oxidation completely. This is because glycolysis generates only 2 moles of ATP, unlike oxidative phosphorylation, which produces 36 moles of ATP for every molecules of glucose consumed. To produce the amount of ATP that is equivalent to oxidative phosphorylation, glycolysis should increase to a rate 18 times of its basal flux. In reality, however, this is impossible because glycolysis, even when maximally stimulated by total cerebral ischemia, can only be increased 4-5

fold (Vannucci, 1992). This limited increase may be related to production of H^+ ions that inhibits phosphofructokinase. Indeed, following asphyxic period longer than 10 min, pyruvate level was decreased indicating that there is an early alteration of glycolysis. The rapid change in pyruvate level may also be related to conversion of pyruvate to lactate via lactate dehydrogenase reaction. Two other reasons may also cause reduction of pyruvate level following moderate and severe asphyxia. The first one is, a rise in pyruvate level in the early asphyxia can activate two main anaplerotic enzymes, alanine aminotransferase (Ala-AT) and pyruvate carboxylase which utilize pyruvate to maintain the Kreb's cycle pool. The second one is, an exhausted reserves in substrates suitable for anaerobic energy production (Erecin'ska and Silver, 1989; Lutz, 1992).

The fall in pH was closely correlated with increase in lactate level, both in brain and subcutaneous tissue. Indeed, an increase in lactate level preceded the drop in pH observed in brain and heart tissue, which is in agreement with the idea that anaerobic glycolysis is the dominating cause of acidosis during asphyxia (Siesjö, 1988; Vannucci, 1992). The first sign of an impairment in acid-base homeostasis was observed in lactate level monitored by subcutaneous microdialysis, as early as 2-3 min following asphyxia. Although the origin of lactate measured from subcutaneous tissue by microdialysis is not clear, the level reported here is by large in agreement with that reported in other studies (Rehncrona et al., 1980; Gutierrez et al., 1994; Herrera-Marschitz et al., 1996). Striatal level was also similarly affected, although changes could not be seen before 5-6 min asphyxia. The increase in lactate level observed following mild asphyxia was quite remarkable compared to pyruvate, probably reflecting activation of phosphofructokinase to compensate a deficit in ATP, as well as activation of lactate dehydrogenase. The fall in brain tissue lactate level after 20 min asphyxia may be explained by the hypercapnia that leads to a decrease in glucose consumption (Folbergrova et al., 1972b), resulting in a progressive decrease in glycolytic metabolites. Furthermore, these groups of pups were so severely asphyxiated, that perhaps they had to utilize the existing lactate

as an alternative substrate for energy metabolism (Schurr et al., 1988), which may also lead to reduction of lactate level. Indeed, it has been shown that uptake of lactate by neurons for energy metabolism is enhanced with decreased pH because of lactate/proton cotransport (Dringen et al., 1993).

5.3.2 Changes observed in the levels of pyruvate and lactate after 1-8 days

In subcutaneous tissue, the slight decrease in pyruvate level and the significant decrease in lactate level observed with age in control pups indicate a normal maturation process of the energy metabolizing enzymes. In asphyxic pups the initial rise in the levels of pyruvate and lactate obtained during the immediate post-delivery period (40-80 min) were not observed when investigated 24 hrs following delivery. This indicates that the acute systemic biochemical disturbance that occurred in the periphery could be corrected in less than 24 hrs. The levels of pyruvate and lactate then follow the normal development, as that of the controls at postnatal day 1-8. As in the periphery, striatal pyruvate level correspondingly decreased in both control and asphyxic pups following 24 hrs and made age related changes thereafter. In contrast, lactate level increased in control and mild asphyxia, but decreased in moderate and severe asphyxiated pups following 24 hrs after delivery. The increase is perhaps related to increased glucose utilization and lactate production as a result glutamate uptake induced increase in aerobic glycolysis since the level of glutamate decreased in these pups after 24 hrs. (Pellerin and Magistretti, 1994). In moderate and severely asphyxiated pups, however, the decrease may be attributed to an increase in its metabolism as an alternative substrate for energy production (Schurr et al., 1988). Lactate started to drop significantly in asphyxiated pups following postnatal day 4. This is probably associated with the physical maturity of the rat pups. As age increases energy demand also increases, therefore, the accumulated lactate might be vigorously used to meet this demand. Schurr et al. (1988) have shown that lactate is not strictly an end

product of energy metabolism, but rather a secondary energy substrate which under certain circumstances can become a principal energy source.

5.3.3 Changes in the levels of glutamate and aspartate after 80 min

The existence of two or more metabolically distinct pools of glutamate in the CNS was first suggested by Berl and Clark (1969). Fonnum (1984) has estimated that the transmitter pool accounts for 20-45% of the total glutamate found in the CNS. In addition, there is also a possible diffusion of the amino acids from the metabolic to the neuronal pool (Laake et al., 1992; Schousboe et al., 1993). Therefore, in most cases it is difficult to exactly point out the pool the measured amino acids represent. In the periphery, extracellular glutamate and aspartate levels probably represent metabolic pool, since they have been shown to participate in mitochondrial oxidative phosphorylation in skeletal muscle (Blanchaer, 1964; Gutierrez et al., 1994). However, they might also originate from nerve terminals, since glutamate and aspartate like immuno-reactive fibers have been described in the region, where the subcutaneous microdialysis probe was implanted (Nordlind et al., 1993). In the striatum, glutamate has been described together with aspartate, in terminals from corticostriatal and thalamostriatal pathways (Druce et al., 1982; Sandberg et al., 1985; Girault et al., 1986; Palmer et al., 1989). Nonetheless, an intrastriatal glutamate-independent aspartate system has recently been described (Pettersson et al., 1996). Therefore, striatal glutamate and aspartate levels could represent different neuronal pools.

Although glutamate synapses are probably immature in the newborn period, certain regions in the rat brain can have an ample supply of specific glutamate receptor binding sites by 7 days of age. For example, the immature corpus striatum already has the same concentration of glutamate receptors as the adult animal at this age (Johnston and Silverstein, 1987). Hence, though the synapses in the newborn pups are immature and not many, they may release certain

amount of glutamate required for normal development (see Teng et al., 1996). Therefore, whatever pool (neuronal or metabolic) the measured aminoacids represent (see Schousboe et al., 1993), it seems likely that, it is because of the immaturity of the synapses and metabolizing enzymes that it was not possible to observe greater changes in the levels of glutamate and aspartate in this study during the 40-80 min period

As reported previously (Dell'Anna et al., 1995), it was observed that the levels of subcutaneous glutamate and aspartate were increased following mild asphyxia. Moreover, the levels of brain tissue glutamate and aspartate were also increased by 10-11 min asphyxia. Transient increase in EAAs has been observed in several experimental models of asphyxia, and is implicated as a cause of cytotoxic cascade leading to brain damage (Barks and Silverstein, 1992; Johnston, 1993). The transient increase observed in the present study may also be responsible for immediate and delayed neuronal changes observed after mild asphyxia (Dell'Anna et al., 1995, 1996). Several explanations are possible for the transient increase: (i) inhibition of the ATP-dependent reuptake pump for the amino acids (Natio and Ueda, 1983, 1985); (ii) hydrolysis of adenine nucleotides leads to a rise in the concentration of inorganic phosphate, that stimulates the activity of glutaminase; (iii) a rise in concentration of pyruvate on activation of glycolysis could enhance alanine production via the Ala-AT reaction leading to an increase in glutamate synthesis (Erecin'ska and Silver, 1990; Schousboe et al., 1993); (iv) early break down of adenine nucleotides perhaps lead to a rise in ammonia which could contribute to enhancement of glutamine synthesis and reductive amination (Schultz and Lowenstein, 1978).

The observed decrease in glutamate and aspartate with the length of asphyxia probably reflects inhibition of glutamate synthesis by the extreme anaerobic condition, particularly by strong inhibition of glutaminase when the pH falls below 7. Whether this constitutes a protective mechanism against over production of the EAA transmitters remains to be established (Erecin'ska and Silver, 1990). Although the shift in aspartate amino-transferase (Asp-AT)

reaction ought to increase glutamate concentration initially, later glutamate may be lost by decarboxylation to gamma aminobuytric acid (GABA), or oxidation via the glutamate dehydrogenase reaction (Seisjö, 1978). This, latter reaction may also be responsible for the reduction in aspartate level observed in late asphyxia. In addition, the striatal aspartate system, described above, may particularly be sensitive to the asphyctic insult leading to inhibition of its synthesis (Lutz, 1992).

5.3.4 Changes in the levels of glutamate and aspartate after 1-8 days

Although there was a decrease in subcutaneous levels of glutamate and aspartate at postnatal day 1, a significant reduction was observed at postnatal days 4-8, probably reflecting a substantial increase in enzyme activity during development and restoration of normal metabolic processes. In contrast, the levels of glutamate and aspartate in brain tissue, at postnatal days 1-8 were almost identical in all pups, indicating that glutamate metabolism recovers almost immediately to maintain glutamate homeostasis. The concentration of glutamate in the CNS is low as compared to that in other organs (Erecin'ska and Silver, 1990, Herrera-Marschitz et al., 1996) and there is a strict and complicated control of production and release of glutamate (Schousboe et al., 1993). Therefore, once the insult is over, it appears that every mechanism comes in to play to keep the pace of glutamate metabolism with development.

5.3.5 Changes in the levels of monoamines after 80 min

Following 80 min after delivery, the levels of monoamines were increased as early as 2 min after onset of asphyxia. However, the level of dopamine decreased when asphyxia was longer than 16 min which is in line with other studies (see Obrenovitch and Richards, 1995). It is worth recalling that striatal glutamate also decreased gradually when aspyhxia was prolonged, but much more slowly than dopamine, probably because of progressive glutamate leakage from its

large metabolic pool. The central dopaminergic system in the basal ganglia play an important role in the control of motor behavior in rat (Ungrestedt, 1980). Thus, the behavioral changes particularly, locomotion and motility, observed in the present study may be related with the reduction in dopamine turnover in moderate and severely asphyxiated pups. Therefore, this may indicate that 16-19 min of asphyxia seems likely to be the critical time interval to induce long-lasting functional changes in the meso-striatal dopamine system. It has been shown that dopamine receptors become functional during the prenatal period, from embryonic day of 17 (Andersson and Gazzara, 1993) and severe hypoxia/ischemia results in loss of D_1 and D_2 (Kostic et al., 1991). Thus, changes in dopamine receptor function may also contribute to the reduced locomotory activities observed following moderate and severe asphyxia.

The increase in the levels of monoamines on asphyxia was not, however, matched by a similar increase in the levels of their deaminated metabolites. This may in part be attributed to decreased monoamine oxidase (MAO) activity under the hypoxic conditions (Obrenovitch and Richards, 1995). It is believed that DOPAC is predominantly formed from newly synthesized stores of dopamine indicating the rate of dopamine synthesis, while HVA largely reflect metabolism of the released dopamine (Arbuthnott et al., 1990),.

Massive release of monoamines during asphyxia may not be, by itself, responsible for initiation of neuronal damage. Monoamines, or their metabolic by-products, however, may become neurotoxic. Monoamines are readily oxidized, both enzymatically via MAO, and by molecular oxygen, generating potentially neurotoxic oxygen free radicals that have themselves been implicated as mediators of neuronal death (Obrenovitch and Richards, 1995). In addition to its neuromodulatory properties, serotonin is also a potent vasoconstrictor, which may further decrease blood flow in ischemia aggravating ischemic neuronal damage (Obrenovitch and Richards, 1995). Therefore, the acute increase in monoamines observed in this study may indicate a neurotoxic potential of monoamines and their deaminated metabolites. Indeed, it has

been suggested that after carotid ligation and exposure to 8% oxygen, acute release of dopamine from immature nerve terminals may be closely associated with the threshold of hypoxia-ischemia sufficient to initiate brain injury (Johnston and Silverstein, 1987).

5.3.6 Changes in the levels of monoamines after 1-8 days

After postnatal day 1-8, there was an increase in the levels of monoamines, which could be associated with development. However, the increase in dopamine in the most severely asphyxiated pups was less pronounced than the short asphyxiated and the controls, which shows that perinatal asphyxia can produce a long-lasting reduction in the activity of the meso-striatal dopamine system.

5.4 Role of hypothermia and plant extracts on survival

When asphyxia was induced under hypothermic condition, there was an increase in the rate of survival; profound hypothermia providing better protective effect compared to moderate hypothermia. This is in good agreement with studies conducted to show what degree of hypothermia is necessary to offer neuroprotective effect in neonatal experimental rats (Young et al., 1983). The slope for the survival curve at 37°C was steeper than that of 30°C and 15°C, which was intermediate and less steep respectively. The steeper the slope, the greater the change in survival rate with a small increase in time. Thus, at 37°C, survival decreased very rapidly with increasing time after 16 min and came to nil at 22 min even though the increase in time was smaller. In contrast, at 30°C and 15°C survival declined slowly over a period of time.

The underlying mechanism for the neuroprotective effect of hypothermia is not well understood, but different studies conducted so far implicate the following mechanisms; (i) decrease in energy demand by slowing metabolic reactions; (ii) left ward shift of

oxyhemoglglobin dissociation curve; (iii) decrease in EAA efflux (Seisjö, 1978; Lo et al., 1993); (iv) reversal of the postischemic inhibition of protein synthesis (Widmann et al., 1993); (v) increased ubiquitin synthesis and protein ubiquitination (Yamashita et al., 1991); (vi) delay in calcium accumulation (Akira et al., 1991; Bickler et al., 1994); and (vii) membrane stabilization or sealing (Urenjack and Obrenovitch, 1996). Although different mechanisms have been proposed for the protective effect of hypothermia, all of them support the idea that hypothermia slows down metabolic rate, thereby reducing the over all oxygen requirement.

Although there is no empirical information on the use of a particular plant for perinatal asphyxia, an attempt was made to carry out a pilot experiment on certain plants which were believed to have certain protective effect. However, the outcome was not as expected. Amongst the screened plants, only *Heliotropium longifolium* seemed worth considering for future inves-tigation. The others did not show any significant effect. The effect of *Heliotropium longifolium* is perhaps associated with its flavonoids which may have free radical scavenging property.

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DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in any other University and that all the sources of material used for the thesis have been duly acknowldged.

Name EPHREM ENGIDAWORK Signature

Place of submission ADDIS ABABA Date June 10th /1992