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Preclinical Appraisal of Terbutaline Kinfolks in Precipitation of Autistic Spectrum Disorder

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Abstract

Terbutaline is β_2 agonist used in the clinical management of asthma and as a tocolytic agent during pregnancy. In the recent past, preterm use of terbutaline has revealed to hasten autistic like symptoms in the offspring's. In view of the same the present study was inquested to appraise the effect of pharmacological siblings of terbutaline (salbutamol, salmeterol and montelukast) in progression of ASD's in experimental animals. Pregnant rats were treated with salbutamol (10 mg/kg, sc), salmeterol (10 µg/kg, sc) and montelukast (10 mg/kg, sc) and the offspring's were scrutinized for the behavioral, biochemical, neuro-inflammatory and histopathological changes. The offspring's from the rats treated with salbutamol and montelukast were beheld to be closely associated with various symptoms of ASD's.

Key words: Terbutaline, autistic spectrum disorder, salbutamol, montelukast, salmeterol, prenatal

Introduction

Autistic spectrum disorders (ASDs) are the cluster of neurodevelopmental disorders in the category of pervasive developmental disorders, delineated by impaired social interaction, communication, restricted and repetitive behavior. ASD's are perceived in all population with a prevalence of 6 cases per 1000, with about four times more males diagnosed then females.[1]As evident through literature, the pathophysiological changes in brain begin during fetal development in autistic individuals.[2] Contemporary studies prompted that the maternal exposure to illness, medication, environmental toxins and psychological stress are concorded with the prevalence of ASDs.[3]

Terbutaline is a β_2 adrenergic receptor (B2AR) agonist used for asthma and also as a tocolytic agent during preterm labor. Terbutaline readily crosses the placenta, and can disrupt the replication and differentiation of developing neurons. The progeny of pregnant women treated with terbutaline are manifested with impaired school performance, cognitive dysfunction and inflated incidence of psychiatric disorders. A recent study accompanied at Duke University exemplified the augmented risk of brain damage and cognitive defects in babies whose mothers were exposed to terbutaline during pregnancy.[4]Preclinical studies have also deliberated terbutaline as a neuroteratogen that alters neuroprotein markers, architectural organization along with neuronal and glial cells distributions in the cerebellum, hippocampus and somatosensory cortex.[4] Following the same strategic path, Zerrate et al validated innate neuroinflammation after terbutaline treatment, as delineated by microglial activation and behavioral anomalies.[5]

In 2011, FDA began necessitating a black box warning for terbutaline, stating that the drug should not be administered to pregnant women. Terbutaline actuates B2AR receptors and dysregulates the adenyl cyclase, leading to anomalous generation of cyclic adenosine monophosphate in different stages of development in the neonate.[3]The mature cells are protected from such overstimulation

due to their competence to uncouple receptors from production of cAMP.[6]ExaggeratedcAMP either through B2AR stimulation or other stress related factors also precipitates the inflated oxidative stress, which over the above push forward the toxicity.

In consideration to the complications associated with terbutaline during pregnancy and recent directions issued by FDA, salbutamol (B2AR agonist) and salmeterol (B2AR agonist) are the drugs of choice to be used for management preterm labor and/or asthma during pregnancy. In addition to the above mentioned, montelukast (a leukotriene receptor antagonist), nowadays is also used for the management of asthmatic condition in during pregnancy (Figure I). Nonetheless, till date no direct or indirect scientific data exists, which can define the safety profile of salbutamol /salmeterol /montelukast in expecting mothers. Eventually, the present study was ventured to investigate the neuroinflammatory, biochemical and behavioral alterations in experimental animals after salbutamol/salmeterol/montelukast exposure.

Animals

Pregnant albino rats (wistar strain) were acquired from central animal house, United Institute of Pharmacy, United Group of Institution, Naini, Allahabad, (U.P), India. The animals were housed in polypropylene breeding cages, under standard condition of temperature (25 ± 1ºC) with 12h light/dark cycle and free access to commercial pellets diet and water *ad libitum*. The experimental protocol was endorsed by "Institutional Animal Ethical Committee (IAEC)" (UIP/IAEC/2014/FEB./04). The study was supervised as per the guidelines laid by Department of Animal Welfare, Government of India.

Drugs

Salbutamol was received as gift sample from Yash Pharma Laboratories Private Limited, Thane, India. Montelukast (Montair, Cipla Global Limited, Mumbai, India), Salmeterol (Esiflow, Lupin,

Laboratories Limited, Mumbai, India) and DPT vaccine (Comvac 3: Bharat Biotech International Limited, Hyderabad, India) were solicited from the local market.

Chemicals

Ovalbumin, aluminum hydroxide, DTNB (5, 5-dithiotris-2-nitrobenzoic acid) and donepezil solution were retrieved from Himedia Laboratories, Mumbai, India. Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Potassium dihydrogen phosphate and di-sodium hydrogen phosphate were supplied by SD Fine Chemicals Limited, Mumbai, India. All other chemicals used were of analytical grade and procured from Hi Media Laboratories, Mumbai, India.

Experimental protocol

Pregnant rats were selected and relocated to the laboratory and allowed to acclimatize in laboratory conditions. Twenty pregnant rats were divided into 5 groups at random (n = 4).The group I and II served as sham control and toxic control consequently. The group III, IV and V were treated and salbutamol (10 mg/kg, sc), salmeterol (10 µg/kg, sc) and montelukast (10 mg/kg, sc) correspondingly. The pregnant females were daily treated with the respective dose for seven days, 30 min before the OVA aerosol challenge. The pregnant rats in control and toxic control were treated with the subsequent volume of normal saline instead.

OVA aerosol challenge

The group II, III, IV and V were sensitized with of 1 ml (sc) saline containing 1mg of ova albumin and 3.5 mg of aluminium hydroxide, in addition 0.5 ml (sc) of *Bordetella pertussis* vaccine containing 2×10^{10} heat killed organism was administered as an adjuvant. The sensitized rats were divulged to aerosol of 1% ova albumin every day from 14 to 21, while the control rats acknowledged filtered air only. Pups (n=8) from the pregnant dams were looked over for post natal growth and behavioral alteration up to 45 PND. On 46^{th} day animals were sacrificed using light anesthesia followed by heart perfusion for removal of total blood from the brain. Brains were

extracted without damage. Collected brain tissues were evaluated on the prototypes of biochemical, oxidative embodiments, inflammatory markers and histopathological changes.

Postnatal growth and maturation development

Pups were evaluated for weight gain on 5^{th},10^{th},15^{th},25^{th}PNDs and for eye opening once daily on PND's 9^{th}, 12^{th} to15^{th} and were rated as follows: 0 for both eyes closed:1- one eye open: 2- both eyes open.[7]

Behavioral tests

Negative geotaxis

Negative geotaxis was contemplated once daily on PNDs 7^{th}, 9^{th} and11^{th}. Pups were timed for completing a 180º turn when placed in a head down position on a 25º inclined surface. Negative geotaxis reflects vestibular function and motor development.[8]

Swimming performance

An aquarium filled with water (28–29 ºC) was used for swimming test on PNDs 10^{th}, 12^{th}, 14^{th}, and 16^{th}. Each animal was put at the center of the aquarium andperceived for 5–10 sec. The swimming performance was figured out according to the position of nose and head (angle) on the surface of water. The angle of swimming was appraised as follows: 0- head and nose below the surface; 1- nose below the surface; 2- nose and top of head at or above the surface but ears still below the surface; 3- same as two except that water line was at mid-ear level; and 4- same as three except that water line was at the bottom of ears. Swimming is a computation of motor development and consolidation of coordinated reflexes responses.[9]

Locomotor activity

It was documented individually for each animal by numbers of photo beams crossings in actophotometer in 10 min of interval.[10]

Olfactory discrimination

The test was conducted on PNDs 10th, 12th, and 15th. The apparatus dwelled of a plastic container 20× 8× 8cm^3 (l× w× h), two small bins, and a clear plastic cover, which was placed over the bins and container. One end of the container encompasses a bin filled with new bedding, while the other end had a bin filled with home cage bedding. A 3 cm^{2}of area was demarcated at the center of the screen. A line was drawn on the screen above each bin. Each pup was allocated in the centrally marked off region on the screen, and the latency to enter the home bedding side by crossing the devised line with the front paws and head was timed. Central placement of the pup was balanced by altering the pup facing to or away the experimenter. Age of home bedding was balanced across the groups and averaged three days old at testing. The test reflects a nest-seeking response arbitrated by the olfactory system.[11]

Nociception

Nociceptive effect was taken account of using tail flick test on PNDs 10th, 15thand 25th using a tail flick analgesiometer. Animal was gently restrained, and radiant heat was focused onto its tail. The cutoff time was 9 sec. Tail flick measurements were taken three times at 30 sec intervals.[12]

Elevated plus maze test (EPM)

EPM was implemented on PNDs 14th, 22thand 30th.The apparatus consist of two open arms (30×5 cm) and two closed arms (30×5×25 cm) in perpendicular position. The open and closed arms were connected by a transparent acrylic. The floor was made of black acrylic. The maze was 45 cm above the floor. The rat was placed at the center of the plus maze with its nose in the direction of one of the closed arms, and contemplated for 5 min for the measurement of the following parameters: number of entries in the open (NEOA) and closed (NECA) arms.[13]

Biochemical changes

The animals were scarified using light ether anesthesiasupersededby heart perfusion on PNDs 45. The brains were evacuated instantly, rinsed with ice cold saline, dried on filter paper and 10 % w/v

tissue homogenates were prepared in ice cold 0.15 M KCI using tissue homogenizer. The post nuclear fraction obtained by centrifuging the homogenate at 10000 rpm for 10 min at 4^{o}C in a cooling centrifuge were used for the spectrophotometric estimation of acetylcholinesterase(AchE),thiobarbituric acid reactive substances (TBARS),catalase, superoxide dismutase (SOD),protein carbonyl and tissue glutathione by using the methods previously established at our laboratory.[13-16]

Inflammatory markers

The level of interleukins IL-1β (catalogue no. K0331212P), IL-2 (catalogue no. K0332100P), IL-4(catalogue no. K0332133P), IL-6(catalogue no. K0331229P), IL-10(catalogue no. K0332134) were enumerated in the brain tissue using Elisa plate reader (Alere AM 2100 microplate reader) from commercially available kits procured from Koma Biotech, Australia.

Morphological Evaluation

Braintissues were appraised histopathologically using hematoxyline and eosin staining. The tissues were fixed in paraformaldehyde for overnight, succeeded by 70% isopropanol overnight. Tissues were farther exposed to augmenting concentration of isopropanol (70%, 90%, and 100%) consequently superseded by dehydration with 100% xylene. The tissues were embedded in paraffin wax and 5µm sections were prepared using microtome followed by staining with hematoxyline and eosin.[17]

Statistical analysis

All data were presented as mean± SD and analyzed by one-way ANOVA followed by Bonferroni test for the possible significance identification between the various groups. $^{c/}$*P< 0.05, $^{b/}$**P< 0.01, and $^{a/}$***P < 0.001 were considered as statistically significant. Statistical analysis was performed using Graph Pad Prism software (3.2), San Diego, California.

Results

Postnatal growth and maturation

Compelling delay in the post natal growth and maturation development was bestowed in all the groups when correlated to sham control (figure II A and B).

Behavioral tests

The pups from the dams treated with drugs in question and ova albumin flaunted momentous subsidence in locomotor activity, which was successively headed towards normalization with progression of age. No significant variation could be observed between the test groups and ova albumin. Similar pattern of restoration was checked out in pups on negative geotaxis prototype with the drugs in questions giving a significant cut back time to align on a negative geotaxis paradigm in comparison to ova albumin treatment (Figure IIC and F). The animals of control group exhibited downturn in innate escape response with upturn in growth. Similar pattern of cutback was ascertained in toxic group and test groups. Nonetheless, salbutamol and montelukast treated groups were perceived to have somewhat higher innate escape response in contrast to control. Thermal nociception was momentously heightened in test groups in comparison sham control; nonetheless detainment in response was contemplated with progression of age. Treatment with salbutamol and monteleukast was observed to afford significant protection against the same in comparison to ova albumin treatment on PND 25 (Figure IID). The ontogeny of swimming behavior was significantly delayed in toxic animals. The drugs in question significantly restored the swimming performance score with montelukast demonstrating to be most efficacious on PND 25 (Figure IIE). When perceived for anxiety like behavior through plus maze, adolescent as well adult pups manifested diminished open arm entries after the ova challenge. The consequential treatment with salbutamol, salmeterol and montelukast further illustrated abatement in number of open arm entries (Figure IIG). The nest seeking behavior arbitrated through olfactory clue was

adversely affected in the toxic group. Successive treatment with salbutamol and montelukast also subsided the nest seeking behavior (Figure IIH).

Biochemical tests

The brain tissues were over the above appraised for the antioxidant defense through embodiments of peroxidation and antioxidant enzymatic defense. Momentous augmentation in TBAR's generation and protein carbonyl formation was contemplated in toxic control which was besides alleviated after the administration of drugs in question. The enzymatic activities of SOD and catalase were noted to be truncated notably in toxic treatment and furthermore diminished after treatment with test drugs. When additionally inquested for AchE activity, we perceived momentous increase in enzymatic activity in the toxic group. Sub sequential administration of test compounds restored the enzymatic activity of AchE slightly (Table-I).

The brain tissues were farther scrutinized for the presence of cytokines pertaining to inflammation and adaptive immunity. The tissue levels of IL-2 and IL-4 were perceived to be consequently inflated after the treatment with test drugs. The levels of pro-inflammatory cytokines, IL-1β and IL-6 were looked out to have compelling upsurge, whereas the IL-10 (anti-inflammatory) levels were curtailed after the administration of drugs in question (Table II).

Histopathology

When contemplated histopathology, serve neuronal degeneration and neuronal loss was observed in the test groups when treated with salbutamol and montelukast. However, salmeterol depicted only slight variability in comparison to control (Figure III).

Discussion

Autism is a neurodevelopmental disorder symbolized by aberrant development, abnormality in social interaction, restricted interest, stereotyped repetitive behavior besides other neuroanatomical, biochemical and inflammatory disturbances. The present probe, reports the

effect of B2ARs agonist (salbutamol and salmeterol) and leukotriene antagonist (montelukast) on amelioration of autistic spectrum disorder in the offspring's of pregnant wistar rats. In order to mimic the clinical condition the rats were challenged with ova albumin and subjected to treatment with salbutamol, salmeterol and montelukast. The pups delivered were consequently contemplatedfor maturation development, sensory function, behavioral changes, biochemical changes, inflammatory markers and brain morphology.

We perceived no change in delay in maturation development between the toxic treatment and subsequent treatment with drugs in question. The experimental animal proclaimed lower sensitivity to spinal pain, exaggerated time to align on negative geotaxis paradigm (delayed reflexes, motor skills and cerebral integration) and attenuation of coordinated reflexes (swimming performance) when treated with ova albumin and the test drugs. It would be apropos to acknowledge that ova albumin flaunted more striking deterioration of the motor coordination (swimming performance and negative geotaxis) and the same was restored towards normal by the drugs in question. The delayed lower pain sensitivity and diminished reflexes in autistic subjects in extensively deliberated phenomenon in clinical and preclinical subjects.[5, 7, 18-19]

Exploratory behavior is one the basic form of behavioral activity and its prenatal disruption can cause CNS developmental damages and autism.[7, 20]Momentous downturn in the locomotor activity was documented in the ova albumin and test drugs treated animals, with more conspicuous effect by salbutamol. The said decline in the locomotor activity is a well settled phenomenon in the distinct experimental models for autism.[21-22] The diminution in the locomotor activity could be accredited either to the reduced number of purkinje fiber in vermal lobules (associated with reduced exploration) or changes in the neural structures markedly in cortex and amygdale.[23-24]The pups were also ascertained with delayed nest seeking response (entries in the old husk, olfactory discrimination) manipulated to ova challenge and test drugs, contributing to social deficits. The

above findings are in corroboration with the antecedent clinical and preclinical reports.[25-26]The concordance between the prenatal exposure with the drugs in question and autism was moreover in quested on the EPM paradigm accounting the NEOA as a measure for anxiety like behavior. The pups manifested augmented anxiety/lower exploratory behavior in EPM with fewer arm entries in open arm when treated with ova albumin. The exploratory behavior was further diminished by the drugs in question with more pronounced effect by montelukast selectively.

The behavioral abnormalities observed in the current experiment were pronounced with the ova albumin challenge which was restored to some extent by the drugs in question. It would be justified to elaborate that the salbutamol has adversely affected the thermal nociception and motor coordination much adversely then the ova challenge. On the contrary monteleukast augmented much pronounced detrimental effects on the exploratory behavior and social deficient paradigm in comparison to control or the ova challenge.

Prima facie the behavioral abnormalities were more extensive in ova challenge suggesting the rise of abnormal phenotypes by ova albumin which was further deteriorated by salbutamol and montelukast in comparison to salmeterol.

Manifold mechanisms like oxidative stress, membrane lipids anomalies, changes in membrane fluidity, changes in immune and inflammatory response, mitochondrial dysfunction besides abnormal energy metabolism have been contemplated toaid in pathogenesis of ASD.[27-29]The antioxidant defense mechanism in ASD may be down or up-regulated, suggesting altered antioxidant defense mechanism. The triple regulated enzymatic defenses of SOD/catalase/GSH represent cellular oxidative stress or successive compensatory mechanisms.[30-31]Similar lines of results were contemplated in the present study, as contemplated with slackened enzymatic activity of SOD and catalase with consecutive treatment of ova albumin and the same was further slackened the salbutamol and monteleukast treatment. Treatment with ova albumin afforded

significant decline in the levels of GSH, which was restored to some extent by the drugs in question except for montelukast. In discordant to enzymatic defense, the oxidative markers of protein and lipid peroxidation are much more evidently defined both clinically and preclinically, when contemplated for ASD's.[32-34]Similar lines of results were arbitrated in the current study after the ova challenge and the same was more conspicuously pronounced by salbutamol (TBARS) and salmeterol (protein carbonyl). Notwithstanding, significant upregulated protein carbonyl content was contemplated after salbutamol, salmeterol and montelukast treatment when compared with ova challenged.

Several cholinergic abnormalities have been reported in autistic subjects, including reduced nAchR binding, reduced M1 receptor binding and intensified activity of brain derived neurotropic factor infact role of cholinergic system in development of autism is well deliberated.[35]The basal forebrain which is involved in the attention is largely resided with cholinergic neurons and is believed to be immensely abnormal in autistic subjects.[36]The present study also corroborated with the previous reports and demonstrated momentous upsurge in the acetylcholinestrease activity by the group subjected to ova challenge. However, sententious variation was observed in between the treatment groups with salbutamol proclaiming least variability. The results implied the non significant deficit cholinergic innervations in the animals treated with test drugs.

The cytokines play a key role in governance of inflammatory/ immune response in neurological circuits and act as the key regulators for the systemic communication. Cytokines affect the developmental and functional aspects of nervous system and dysregulated cytokine production/signaling/regulation is implicated in wide range of neurological disorders together with ASD's.[37-38]In the current study, we evaluated both proinflammatory (IL-1β and IL-6); anti-inflammatory (IL-10 and IL-4) and immunoregulatory (IL-2) cytokines to give a wider prospect to the study.[39]We perceived noteworthy diminution in the levels of anti-inflammatory (IL-10 and IL-

4) cytokines after the ova challenge, which is in corroboration with the forgoing preclinical and clinical studies.[40] Treatment with drugs in questions further diminished the levels of anti-inflammatory cytokines with more pronounced diminution by salbutamol and salmeterol in comparison to monteleukast. The current consideration could be accounted to the focal brain inflammation most adversely affected by the salmeterol and salbutamol. However, when scrutinized for the proinflammatory cytokines (IL-1β and IL-6), remarkable downregulation in the levels of IL-6 and IL-1β was observed by the ova albumin. However, treatment with drugs in question afforded momentous increase in the levels of IL-6, suggesting precipitation of inflammatory reaction by the test drugs. It would be worth to put on records that montelukast and salbutamol were accorded with much more increase in the levels of IL-6 in comparison to salmeterol. On the contrary, unusual decline in the levels of IL-1β was accredited after the test drugs and the same could be accredited to the fact, that IL-1β is a cytokine expressed very early in immune response and contemplating the long duration of the study one may expect a compensatory decline in the levels of IL-1β to counteract inflammation.

In addition to above when embarked for the level of IL-2, we observed consequential down regulation after ova challenge. IL-2 is a signaling molecule of immune system via direct effect on T cells.[41-42] Diminished levels of IL-2 may reflect the utmost immunocompromised state of the experimental animals following the ova challenge. Treatment with the drugs in question helped to restore deregulated immune system with most conspicuous effect demonstrated by the monteleukast. In view of the dysregulated cytokine profile after consequent treatment with the test drugs and considering the reported inflammatory/immunological disturbance affiliated with ASD's, author are in opinion that all the test drugs have the potential to participate in progression of ASD'S to variable degree.

Neuronal degeneration is an undisputed but barely deliberated phenomenon in term of autism. In recent past Kern et al (2013), summarized the diversified aspects related to neurodegeneration in autism and concluded that the neuronal cell loss, activated microglia, pro inflammatory cytokines and oxidative stress accompany the neurodegenerative changes accounted for breakthrough of autism.[43] When appraised histopathologically, the salbutamol and montelukast treated groups were manifested with conspicuous neuronal loss (less number of neuronal cells) and neuronal degeneration.

As amplified above, authors are in viewpoint that inflammatory dysregulation and oxidative stress as launched by ova challenge in the experiment could precipitate various behavioral abnormalities associated with autism. It is to be added further that the treatment with test drugs didn't affected the maturation development helped to restore few of the end points evaluated in the study. However, while going through behavioral, biochemical and histopathological findings one would like to comment that the all the test drugs can have variable effects on the neurological circuits of a developing brain with salbutamol, moteleukast and to some extent salmeterol can push forward various behavioral symptoms of symptoms pertaining to autism. Anyhow, the same could not be affirmed in case of salmeterol and needs additional investigation.

It would be appropriate to notify that all the test drugs are amply lipophilic in nature and can cross the physiological blood brain barrier and placental barrier, and has the proficiency to adversely affect the fetus if administered prenatally. Nonetheless, antecedent report by Manchee and colleague enumerated low transfer of salmeterol across the placental barrier and the same could be accounted for the diminished toxicological effects as securitized in the current study.[44]

Authors would also like to put on records that monoamine oxidase (MAO) inhibitors have been implicated in the disorders like parkinsonism, who are attributed to have a close confinement with

oxidative stress.[45,46,47] Considering the same, we are in opinion that exploiting MAO inhibitors in ASD's could be an viable research question for future.

From above, author can derive the salbutamol and montelukast can stand forth broad spectrum of biochemical, inflammatory, behavioral and neurological changes when administered prenatally, and can hasten the autistic like symptoms in experimental animals. The result also suggests the existence of wide range of pathological concordance between the salbutamol/ montelukast treated animals and previously vested animal's models for autism. With the observation accorded through present study, we would also like to hypothesize that precipitation of autistic symptoms is not the sole prerogative of drug usage during pregnancy; rather the inflammatory and immunological disturbances during pregnancy and their subsequent modification by various drugs could be one of the reason for the precipitation of such a complex behavioral disorder. In spite of, much additional research is vital to elucidate the molecular basis and clinical implications of salbutamol and montelukast treatment.

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Figure I: Chemical structures of salmeterol, salbutamol and montelukast

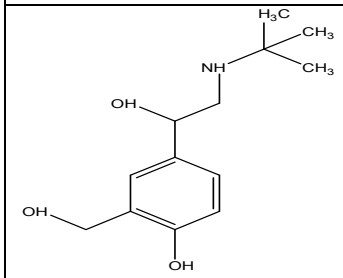
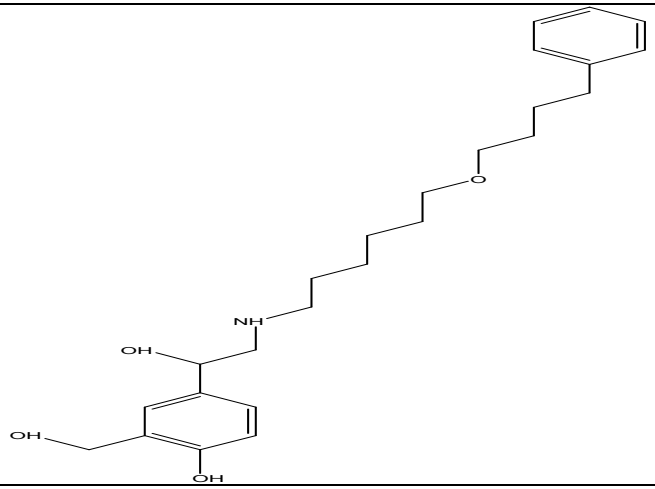
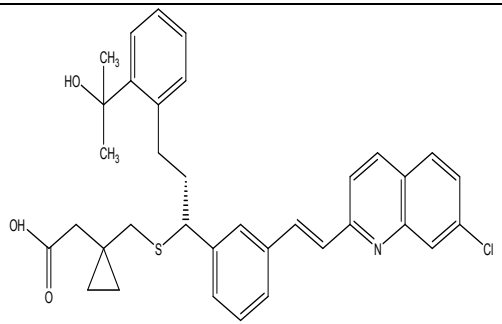
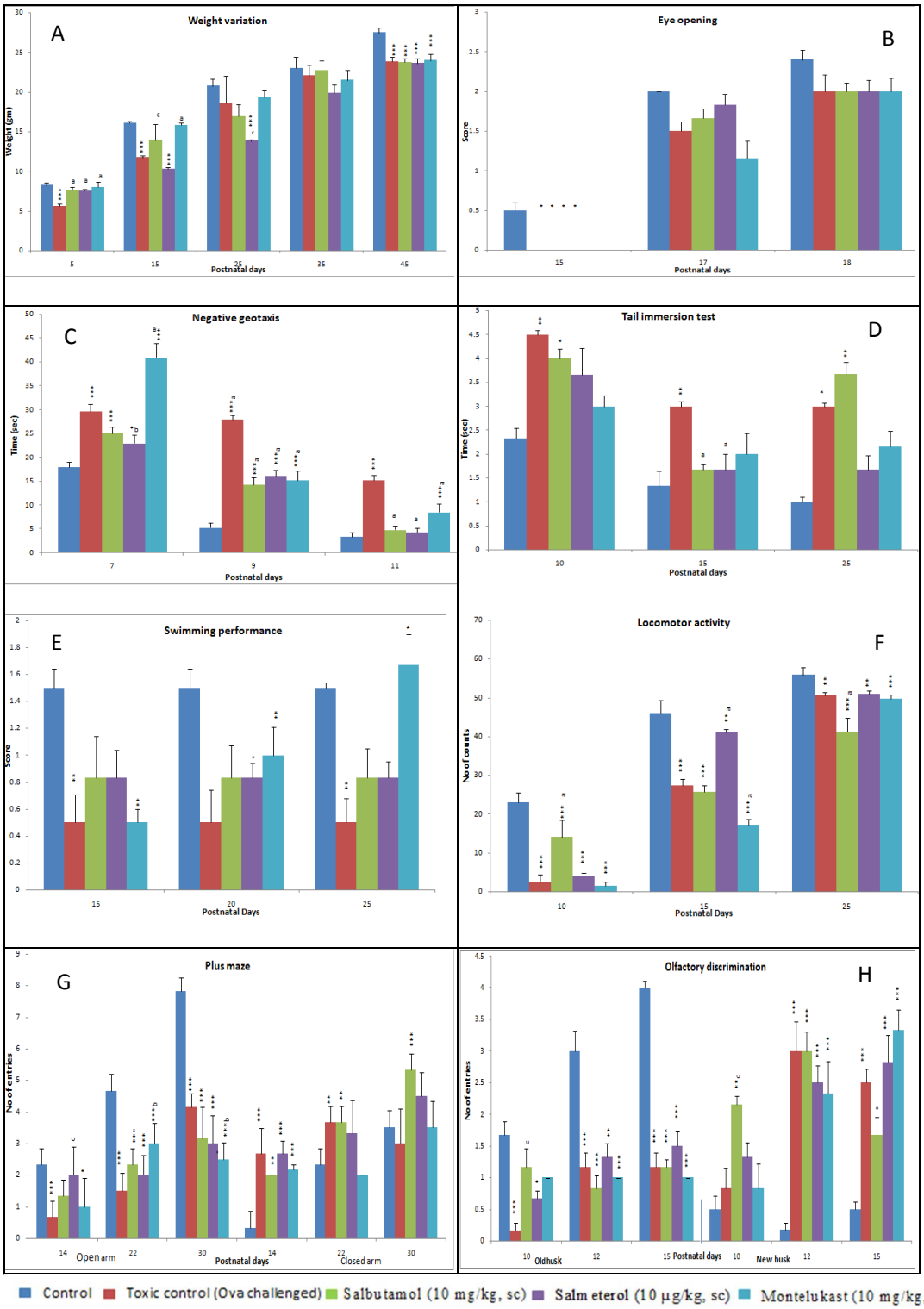
Salbutamol 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol	**Salmeterol** 2-(hydroxymethyl)-4-[1-hydroxy-2-[6-(4-phenylbutoxy)hexylamino]ethyl]phenol	**Montelukast** 2-[1-[[(1R)-1-[3-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-[2-(2-hydroxypropan-2-yl)phenyl]propyl]sulfanylmethyl]cyclopropyl]acetic acid
		

Figure II: Effect of prenatal exposure of terbutaline siblings on maturation development and behavioural changes



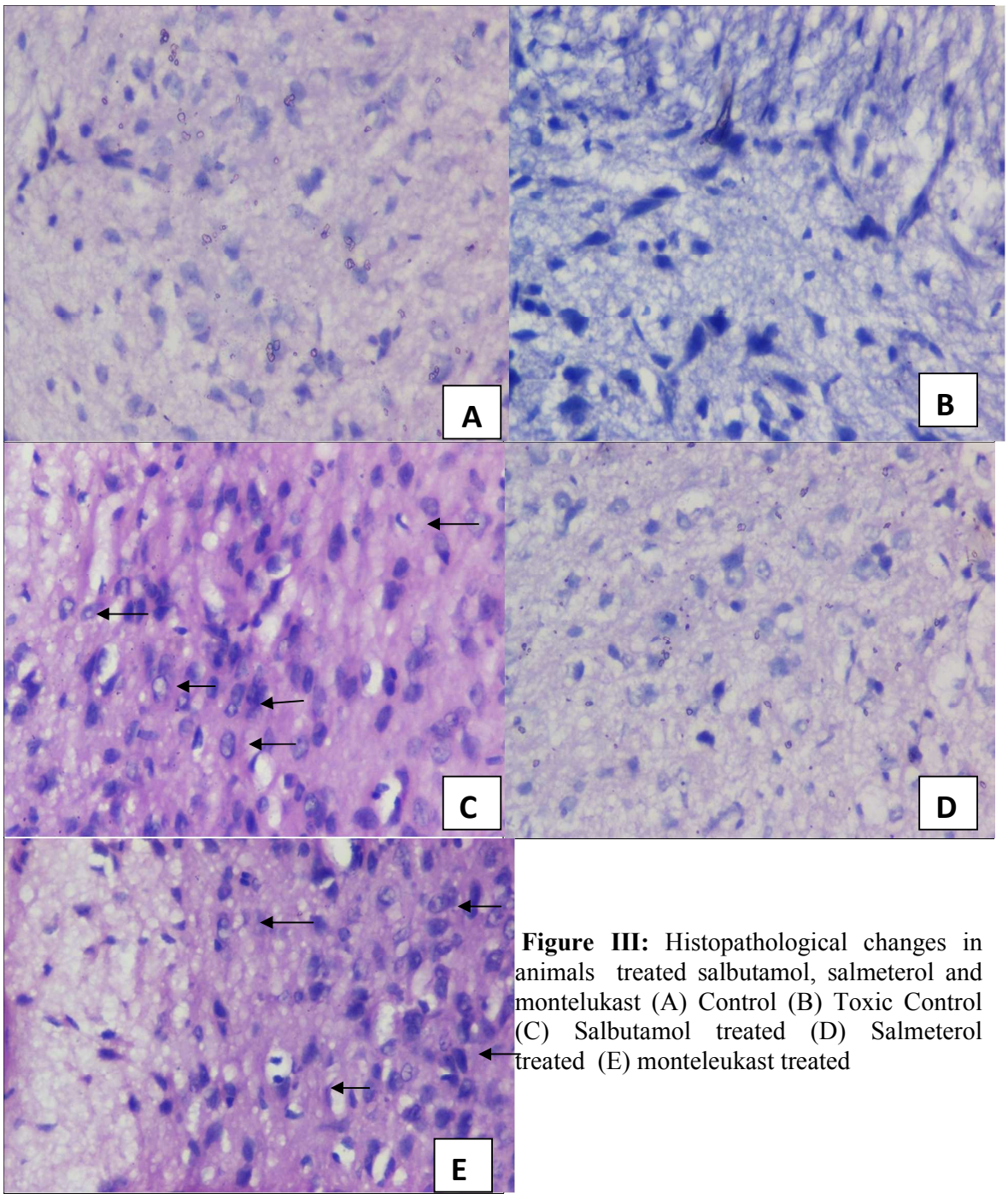


Figure III: Histopathological changes in animals treated salbutamol, salmeterol and montelukast (A) Control (B) Toxic Control (C) Salbutamol treated (D) Salmeterol treated (E) monteleukast treated

Table I: Effect prenatal exposure of terbutaline siblings on physiological antioxidant defence in experimental pups.

Groups	TBARS (Nm of MDA/mg of protein)	GSH*10^{-4} (mg %)	SOD (unit of SOD/mg of protein)	Catalase (nM of H_2O_2/min/mg of protein)	Protein carbonyl (nanomoles /ml unit)	Acetylcholinesterase (Nanomoles/min/ml unit)
I	2.16±0.01	0.25 ± 0.01	0.88 ± 0.01	81.32±2.19	77.42±0.13	0.48 ± 0.03
II	2.73±0.33**	0.14±0.02***	0.77 ± 0.03***	70.15±1.724***	142.35±0.26***	1.07 ± 0.11***
III	4.03±0.20***^{a}	0.19±0.01***^{a}	0.75 ± 0.01***	56.03±2.96***^{a}	146.97±0.52***^{a}	0.62 ± 0.04^{a}
IV	2.87±0.04***	0.19±0.002***^{a}	0.73 ± 0.01***^{c}	62.85±3.34***^{b}	172.65±0.35***^{a}	0.64 ± 0.05^{a}
V	2.28±0.04^{c}	0.16±0.01***	0.77± 0.01***	52.05±1.77***^{a}	164.17±0.47***^{a}	0.67 ± 0.11^{b}

(Values are Mean ± SD); Comparisons were made on the basis of the one-way Anova followed by Bonferroni test.
All groups were compared to the control group (Group 1)(*p<0.05, **p<0.01, ***p<0.001)
Group 3, 4 and 5 were compared to Group 2 (^{c}p<0.05, ^{b}p<0.01, ^{a}p<0.001)

Table II: Effect of prenatal exposure of terbutaline siblings on inflammatory and adaptive immunity markers.

Group	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)
I	386.15 ± 10.34	1579.10 ± 1758.3	1288.9 ± 51.98	789.5 ± 223.11	708.32 ± 28.56
II	92.69 ± 4.8**	898.50 ± 1362.1**	724.95 ± 33.37***	357.62 ± 9.43*	232.68 ± 6.23**
III	294.67 ± 131.58^{c}	1965.30 ± 518.47^{a}	209.03 ± 8.23***^{a}	859.10 ±135.64^{b}	461.06 ± 151.05
IV	279.75 ±7.49^{c}	1469.20 ± 768.90^{b}	199.94 ±15.56***^{a}	857.89 ± 59.735^{b}	365.27 ± 145.50*
V	292.93 ± 7.8^{c}	2978.30±1940.3***^{a}	1166.8 ± 61.34*^{a}	1180.1 ±22.63*^{a}	443.36 ± 11.88

(Values are Mean ± SD), each group contains 8 animals Comparisons were made on the basis of the one-way Anova followed by Bonferroni test. All groups were compared to the control group (Group 1)(*p<0.05, **p<0.01, ***p<0.001) Group 3, 4 and 5 were compared to Group 2 (^{c}p<0.05, ^{b}p<0.01, ^{a}p<0.001)