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IMMUNOHISTOCHEMICAL STUDIES OF BLOOD BRAIN BARRIER AFTER ADMINISTRATION OF ABHRAK BHASM IN WISTAR RATS

Neurology

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ABSTRACT

Abhrak bhasma (AB) is a type of bhasma prepared from repeated incineration of mineral mica with decoctions of about 72 herbs. The particle size of Abhrak bhasm has been shown to be in the range of 29-88 nanometers and Fe, Ca, Si, Mg and K are found to be as major constituent. Many drugs developed to treat Central Nervous System (CNS) disorders are unable to reach the brain parenchyma in therapeutically relevant concentrations. The blood brain barrier protects brain parenchyma from the fluctuation of plasma composition, from pathogenic agents and maintains homeostasis of the brain parenchyma by restricting non-specific flux of ions, peptides, proteins and even cells into and out the brain. Immunohistochemistry is being widely employed as a tool for biological studies. This study is conducted to examine the change in the continuity of Blood brain barrier by using immunohistochemistry, once Abhrak bhasm drug is given in experimental animal and also to examine the histology of organs. In this study a total of 30 adult albino Wistar rats of approximately 4 months age (approx. 150-200 gms) of either sex selected randomly to see the effect of Abhrak bhasm, an ayurvedic drug on Wistar rats. The rats were weighed, marked and divided into 5 groups each consisting of six animals. In normal control group (Group E), no drug was administered and in rest of the four treated groups (Group-A,B,C,D), Abhrak bhasm @ 36 mg/kg B.wt. was administered orally once in each rat. Brain, liver, kidneys,spleen and blood samples were collected in 10% formalin solution after euthanizing the rats at 0.5,2,6 & 12 hours of Abhrak bhasma drug intervention. The alterations in any of the biochemical parameters are within the tolerable limits of liver and kidney since the dose of abhrak bhasm did not affect liver and kidneys. In the present study, the increase in ALP level may be the result of alterations in metabolisms that occurred without any significant alteration in histology of liver. After applying the immunohistochemistry with the research markers GFAP, CD 34, S 100, GLUT-1 and RECA-1 on the rats in groups A,B,C and D, there was no change in the intensity of immunohistochemistry, with respect to control. While on applying the Occludin, the intensity of immunohistochemistry was reduced in all the treatment groups as compared to the control group. On the basis of findings of present study it can be concluded that the therapeutic dose of Abhrak bhasma causes changes at the level of tight junctions present in blood brain barrier in rats which is shown by immunohistochemistry with occludin research marker. There is no toxic effect of drug on different organs of rats as no significant changes in histology of organs are seen. More studies need to be done to check the permeability of blood brain barrier for Abhrak bhasma drug, like calculating its concentration in brain tissues and other vital organs of rat.

KEYWORDS

Abhrak bhasma, Blood-brain barrier, Wistar rats and Immunohistochemistry.

INTRODUCTION:-

Herbomineral formulations of Ayurveda, constituting Bhasma as an ingredient, are the superior forms of administration of nano-medicine. Abhrak Bhasma, a herbomineral product of Ayurvedic pharmaceutical, acts on both the doshas (bodily humors) and the disease to arrest the pathogenesis. Abhrak Bhasma is like a supreme ambrosia; it destroys vata (air), pitta (fire), and disease ksaya (phthisis) **(Hareshwar S et al.2017, p.30)**.

The substance obtained by calcinations is known as Bhasma in Ayurvedic medicine. The process of calcination is called PUTA in Ayurvedic treatment. Based on the number of PUTA applied to the bhasma the quality of the medicine will vary. Basically it will be applied from 7 PUTA to 1000 PUTA, where the number shows the repetition of process of calcination which is carried out after collaborating with the medical extracts and dried in direct sunlight. Bhasma stimulates the metabolic reaction of tissue cell **(Gopinath, H. and Shivashankar, M., 2016, p.2375)**.

Abhrak bhasma (AB) is a type of bhasma prepared from repeated incineration of mineral mica with decoctions of about 72 herbs. The particle size of Abhrak bhasm has been shown to be in the range of 29-88 nanometers and Fe, Ca, Si, Mg and K are found to be as major constituent. The quality of Abhrakbhasma differs as per the number of PUTA performed. The sahastraputiabhrakbhasm that undergoes 1000 PUTA is considered to be of finest quality. Different grades of Abhrakbhasma are used in the treatment of a vast range of ailments and also as a constituent of many rejuvenating formulations **(Subedi, et. al., 2017, p.238).**

Many drugs developed to treat Central Nervous System (CNS) disorders are unable to reach the brain parenchyma in therapeutically relevant concentrations. The blood brain barrier protects brain parenchyma from the fluctuation of plasma composition, from pathogenic agents and maintains homeostasis of the brain parenchyma by restricting non-specific flux of ions, peptides, proteins and even cells into and out the brain **(Rubin,L.L. and Staddon, J.M.1999,p.11).**

In particular, drug delivery across the BBB has to be considered within the context of neuroinflammation, a process that may vary in its extent, but that is probably common to all CNS lesions and diseases, including encephalitis, multiple sclerosis, Alzheimer's disease etc. Neuroinflammation is associated with BBB inflammation and potentially with changes in BBB physiology and permeability considering that paracellular and transcellular transport can be amplified in pathological conditions **(Molino, Y. et.al.2014, p.2)**.

Breakdown of the blood-brain barrier (BBB) is an important contributing factor to injury in many brain diseases, including stroke, head injury and brain tumors. A number of different, partially independent components, including the extracellular matrix, tight junctions (TJs),pericytes, and astrocyte endfeet, together with adherens junctions, form junctional complexes and play a central role in the control of BBB permeability and maintenance of cell polarity **(Fernandez-Lopez et.al.,2012,p.9588).**

Immunohistochemistry is being widely employed as a tool for biological studies.The content of different biomarkers in the blood and cerebrospinal fluid are one of the most informative biochemical indicators of the state of the blood brain barrier (BBB). The neuron-specific proteins (NSPs) outside the brain may be considered as

markers of a pathological process. To evaluate the state of the BBB, it is rational to use the most studied proteins, viz., the markers of neurons and astrocytes. These are the glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), and proteins of the S 100 family. They do not cross the blood brain barrier (BBB) and practically can not be detected in serum under normal conditions. When BBB permeability is compromised, NSPs penetrate into the peripheral blood and may be measured there. Vascular endothelial growth factor (VEGF) may be important for diagnostics for the evaluation of the state of a major component of the BBB, viz., the endothelial cells. Dynamic assessment of the serum levels of NSPs and the VEGF may be used to evaluate BBB resistance and the severity of CNS lesions **(Bilinov & Terent'ev, 2013, p.159).**

The BBB, in particular, is considered one of the most unique, elusive, and impenetrable barriers in the body and has been studied in conjunction with many diseases such as seizures, Alzheimer's and Parkinson's. The delivery of treatments across such biological barriers provides a unique and powerful pathway for neuronal degenerative therapeutics-the BBB, in this respect, remains a challenge for targeted pharmaceutic delivery. It remains an active area of continuing research. In studying the BBB, IHC is widely used to visualize proteins in a qualitative fashion, giving investigator a valuable tool to survey the distribution of proteins within the brain, specifically it's regions (e.g.-Hippocampus) and sb-regions (e.g.-CA1, CA3, Dentate Gyrus, Perforent pathway of the hippocampus) **(Soans et al.2016,p.1&2)**.

This study is conducted to examine the change in the continuity of Blood brain barrier by using immunohistochemistry, once Abhrak bhasm drug is given in experimental animal and also to examine the histology of organs in Wistar rats after administration of Abhrak bhasma.

MATERIAL AND METHODS:-

Animals:- In this study a total of 30 adult albino Wistar rats of approximately 4 months age (body weight approx. 150-200 gms) of either sex selected randomly to see the effect of Abhrak bhasm. Rats were procured from the Animal Husbandry department of UP University of Medical Sciences (UPUMS), Saifai, Etawah **(CPCSEA registration no.-1087/GO/ReBi/S/ReBi/L/CPCSEA)** and the experiment was started after taking the approval from Institutional Animal Ethic's Committee **(IAEC protocol approval no.-IAEC/02/AH/2019-20 dated 07/03/2020)** of UPUMS. Rats were housed under normal conditions and received a standard food and water ad libitum. An hour prior to experimentation, animals were brought and kept in the experimental room. The rats were weighed, marked and divided into 5 groups each consisting of six animals. In normal control group (Group E), no drug was administered and in rest of the four treated groups (Group-A, B, C, D), Abhrak bhasm @ 36 mg/kg Body weight was administered orally once in each rat **(Table-1).**

Table:-1.Different groups of Wistar rats.

S. No.	Group	Treatment	No. of animals	Sample collection time after drug administration (in hours)
1-	A	AbhrakBhasm	06	0.5
2-	B	AbhrakBhasm	06	2.0
3-	C	AbhrakBhasm	06	6.0
4-	D	AbhrakBhasm	06	24
5-	E	No drug (Control)	06	At the end of study

Test drug:-In this study, highest purified form of Abhrak Bhasm drug i.e. Sahastraputi Abhrak bhasm (subjected to 1000 PUTAS) which is considered to be of finest quality **(Subedi, et.al.,2017, p.238)** was used as the test drug. The drug was given along with 0.5 ml honey.

Animal equivalent dose calculation based on body surface area:
Dose of Abhrak Bhasm in adult human=375mg/60-70Kg Body weight **(Singh, J., 2014.)**

Animal Equivalent Dose (mg/kg) =Human Dose (mg/kg) X Conversion factor

Conversion factor for rats is 6.2 **(Nair,A.B. & Jacob,S.,2016, p.29 and Shin et.al.2010, p.353)**

Therefore, AED (for rats) = 36mg/kgB.wt. orally once.

Abhrak Bhasm was weighed as per the body weight of different rats by using milligram digital balance and a suspension of Abhrak Bhasm was prepared with honey for administration in rats.

Organ samples:- Brain, liver, kidneys and spleen samples were collected in 10% formalin solution after euthanizing the rats with Ketamine overdose administered intraperitoneally.

Histological studies:- The fixed tissues of rats were dehydrated with progressively increasing concentrations of ethanol. The tissues were passed through xylene solution to clear the ethanol and facilitate molten paraffin wax infiltration (55˚C). After that, they were embedded in a wax block. Paraffin sections of 3-4 μm thickness were cut with the rotary microtome and placed on cleaned glass slides. Finally the sections were stained with hematoxylin and eosin. The stained slides were examined using a light microscope where the photomicrographs of the tissue samples were recorded.

Immunohistochemistry of Blood brain barrier (BBB):- It was done by using research markers GFAP, S-100, CD-34, RECA-1, Occludin and GLUT-1.

Tissue processing was done by incubating slides sequentially into 100% Xylene, 50% Xylene, anhydrous ethanol for 15 minutes each, followed by anhydrous ethanol,85% alcohol and 75 % alcohol for 5 minutes each, then washed in distilled water. Antigen retrieval was done by incubating slides with citric acid (pH 6.0) antigen retrieval buffer and microwave for 8 minutes, then slides were washed with PBS (Phosphate Buffer Saline) for 5 minutes. Then slides were incubated in 3 % Hydrogen peroxide solution for 10 minutes at room temperature and washed 3 times with PBS for 5 minutes. Then after drying a PAP pen was used to draw circles around the tissue sections and BSA (Bovine Serum Albumin) blocking was done. After removing blocking solution, solution of primary antibody (Occludin & RECA-1in the dilution of 1:100 and GLUT-1 in the dilution of 1:200) was added on the slide, and incubated overnight at 4˚C.Then after washing the slides the corresponding secondary antibody solution (HRP labelled) was added and the slides were incubated at room temperature for 30 minutes. Then DAB(3,3'-diaminobenzidine) staining was done by adding fresh DAB staining buffer to the circles and after this Haematoxylin staining was done. Then the slides were incubated sequentially into different concentrations of alcohol and after drying the slides were sealed by using neutral gum. The slides were scanned under the microscope and the photomicrographs of IHC findings were recorded.

RESULTS & DISCUSSION:-

Table 2:-Effect of Abhrak Bhasma on Urea, Creatinine, Total proteins, albumins, globulins and serum enzymes in albino rats.

S. No.	Biochemical parameters	Normal range[1]	Control group	Treatment groups				Statistical Value	
				A	B	C	D	P value	Sum of squares(R^2)
2.	Urea	12.3-27.1 (mg/dl)	46.63±2.81	40.73±3.63	40.85±5.40	40.70±3.68	39.62±2.87	0.705	188.009
3.	Creatinine	0.2-0.6 (mg/dl)	0.66±0.17*	0.77±0.05	0.49±0.033[b]	0.66±0.06[b]	0.98±0.11*[b c]	**0.001**	0.782
9.	Total Protein	5.2-7.7 (mg/dl)	8.15±0.41*	5.57±0.17*[a]	5.13±0.31*[b]	7.69±0.62[a b]	7.41±0.29[a b]	**0.000**	43.727
10.	Albumin	3.4-5.5 (g/dl)	4.63±0.22*	3.61±0.13*[a]	3.44±0.12*[b]	4.65±0.34[ab]	3.96±0.28	**0.002**	7.667
11.	Globulin	1.5-2.5 (g/dl)	3.51±0.25*	1.96±0.16*[a]	1.69±0.21*[b]	3.04±0.33[ab]	3.45±0.23[ab]	**0.000**	17.456
12.	SAG Ratio	1.58-3	1.34±0.08*	1.91±0.15	2.27±0.38*[b]	1.57±0.09	1.18±0.13[b]	**0.005**	4.640
13.	AST	65-203 (IU/L)	235.33±17.66	244.00±28.70	249.33±26.61	192.00±24.37	238.83±11.90	0.417	12613.200
14.	ALT	16-48 (IU/L)	103.50±9.64	71.00±4.35	110.00±3.35	122.33±17.63	94.17±7.95	**0.016**	8915.133
15.	ALP	26-230 (IU/L)	238.00±34.95*	231.67±14.13*[a]	601.50±25.51*[ab]	369.83±22.50[b]	258.50±54.38[b]	**0.000**	588327.533

All values are expressed as Mean±SE. Starmark (*) shows the significant association between means of respective group when compared with control at 95% confidence interval (when P<0.05) .

a-indicates significant difference (P<0.05) in the values in between groups as compared to group A.
b-indicates significant difference (P<0.05) in the values in between groups as compared to group B.
c- indicates significant difference (P<0.05) in the values in between groups as compared to group C.
1-Gilkinis, M.L.A. and Clifford, C.B., (2008, p.8&9).

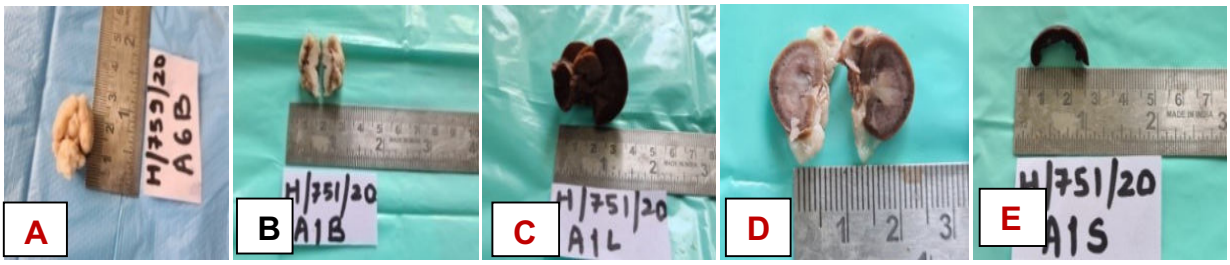

Fig.-1. Gross pictures of different organs of rats: Whole brain of rat with ventral surface (A). Longitudinal cut section of brain (B). Whole liver of rat (C). Cut section of kidney with attached adrenal (D). Whole spleen of rat (E).

Table 3:- Abhrak bhasm mediated variations in the wet weights (mg/gm body weight) of Brain, Liver, Kidney & Spleen of albino rats:-

S. No.	Organs	Control group	Treatment groups				Statistical value	
			A	B	C	D	P value	Sum of squares (R^2)
1.	Brain	12.63±0.36*	12.19±0.52	10.50±0.29*b	12.46±0.56	13.76±0.59b	**0.002**	33.109
2.	Liver	66.69±1.30	64.09±1.70	56.95±2.08b	64.89±2.89	73.84±3.55b	**0.001**	878.767
3.	Kidney	5.37±0.09	5.09±0.14	4.57±0.16b	5.18±0.28	5.96±0.28b	**0.002**	5.992
4.	Spleen	2.24±0.13	3.20±0.49	2.15±0.20	2.80±0.14	3.19±0.28	**0.029**	6.052

All values are expressed as Mean ± SE. Starmark (*) shows the significant association between means of respective group when compared with control at 95% confidence interval (when P<0.05).
b-indicates significant difference (P<0.05) in the values in between groups as compared to group B.

The results of biochemical studies were listed in Table-3. No significant changes were observed in values of Urea, Aspartate amino transferase (AST) and (Alanine aminotransferase (ALT) in treatment groups when compared with control group. However, a significant increase was observed in Serum Albumin Globulin Ratio (SAG) ratio and Alkaline Phosphatase (ALP) in group B i.e. after 2 hours of Abhrak Bhasm administration as compared to control group. There was significant decrease in Total protein, albumin and globulin values in groups A & B i.e. after half an hour and 2 hours of drug administration as compared to control group.

The alterations in the wet weights of brain, liver, kidney & spleen tissues are given in Table-3.The wet weight of brain in control group was 12.63±0.52 mg/gm body weight. There was significant decrease in brain weight after 2 hours of administration of 36mg/kg body weight abhrak bhasm in rats. In control group,the wet weight of liver was 66.69±1.30 mg/gm body weight and wet weight of kidney was 5.37±0.09 mg/gm body weight. However, it was not changed in treatment groups after administration of abhrak bhasm drug. These findings are in accordance with the findings of **Buwa, S. K., (2000, p.258)**, in which the wet weights of liver and kidneys were not changed significantly after administration of different doses 10, 20, 30 & 40 mg/kg body weight of abhrak bhasm drug in different groups of rats. Similarly the wet weight of spleen in control group was 2.24±0.13 mg/kg body weight and it was not changed significantly in treatment groups.

The alterations in any of the biochemical parameters are within the tolerable limits of liver and kidney since the dose of abhrak bhasm did not affect liver and kidneys. In the present study, the increase in ALP level may be the result of alterations in metabolisms that occurred without any significant alteration in histology of liver (**Buwa, S. K.2000, p.253**).

Histological findings:-

In all the treatment groups, histologically, the microscopic features seen in brain, liver, kidney, spleen & adrenal tissues are shown in **figure 2** & **figure 3** and no significant difference was seen as compared to control group. Similar findings were reported by **Buwa, S.K.,(2000,p.256 & p.257)** in a study conducted to see the abhrak bhasma mediated variations in histology and lipases of liver, kidney and adipose tissue of albino rats in which the rats were divided into 6 groups each containing 8 animals. First group was maintained as normal and they were not given any treatment. The animals of 2 to 5 groups were given oral treatment of 10, 20, 30 & 40 mg/kg body weight abhrak bhasma drug for a period of 7 days. At the end of experiments the rats were killed by giving ether anesthesia and the histology of liver and kidney showed normal appearance with healthy cells. No significant change was seen as compared to liver and kidney tissues of control group.

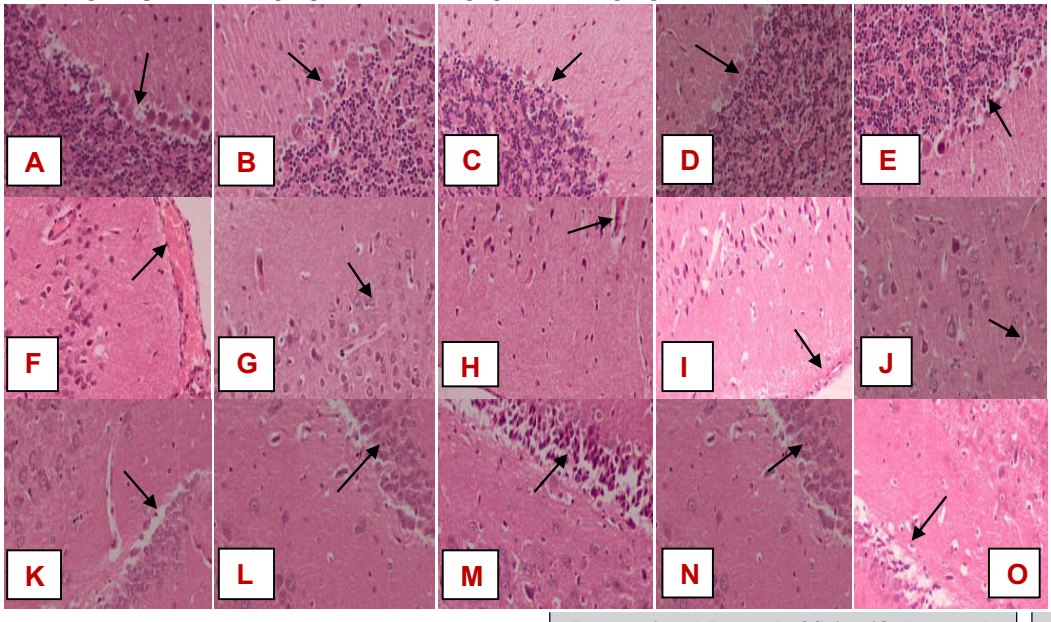

Figure2. Photomicrographs of different areas of rat brain. Figures- A, F and K show cerebellum (organized purkinje cells), cerebral cortex (normal vessels) and hippocampus (unremarkable C4 and dentate gyrus) respectively in rat of control group. Similar findings were seen in group A (figures- B, G and L); in group B (figures- C, H and M); in group C (figures- D, I and N) and in group D (figures- E, J and O) **(as shown by arrows)**. No change were seen in all the treatment groups as compared to Control group. (H&E 40X).

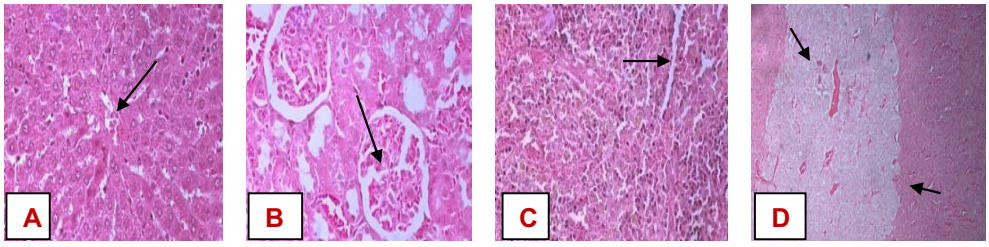

Fig.3 Photomicrographs of histology of rat liver (Control group as well as treatment groups show similar findings like normal hepatic cords, hepatocytes, sinusoids & bile canaliculi) 40X **[A]**; kidney (Control and all treatment groups show no change in glomerular basement membrane, bowman's capsule, proximal convoluted tubule, distal convoluted tubule, medullary region and interstitium) 40X **[B]**; spleen (Control group and all treatment groups show mildly expanded red pulp filled with blood and atrophied white pulp) 40X **[C]** and adrenal(Control and all treatment groups show unremarkable cortex and medulla)(as shown by arrows) 20X **[D]. (as shown by arrows)**

Immunohistochemical findings for Blood brain barrier:- After applying the immunohistochemistry with the markers GFAP, CD 34,S 100, GLUT-1 and RECA-1 on the rats in groups A,B,C and D which is given a dose of Abhrak Bhasm @ 36 mg/kg Body weight at a duration of half an hour, 2 hours, 6 hours and 24 hours, there was no change in the intensity of immunohistochemistry, with respect to control. While on applying the Occludin **(figure-6)**, the intensity of immunohistochemistry was reduced in all the treatment groups as compared to the control group. The occludin antibody in different areas of brain like cortex, hippocampus, cerebellum and choroid plexus show moderate to weak intensity of immunoreactivity in both , blood vessels and capillaries in all treatment groups as compared to control group. In our study we also found that cortex showed heterogenous intensity of immunoreactivity in which frontal region slightly weaker intensity as compared to occipital region in all treatment groups which means there must be some molecular changes at the receptor level due to administration of Abhrak bhasm at different interval of time, either blockage of receptor or less likely damage of receptor. Hence occludin could not bind the receptor and finally the intensity is reduced. This finding is in support of the findings in the literature of **Pan et al., (2017, p.1)** according to which Occludin is a tight junction protein that is a key structural component of the BBB. Degradation of occludin is frequently seen in ischemic stroke and contributes to BBB disruption. Several studies have reported that the alteration or decrease in occludin during cerebral ischemia results in increased BBB permeability **(Kim et al.2020, p.2)**.

Strong immunoreactivity of blood vessels of cerebellum, cortex and hippocampus in all groups are seen while applying RECA-1 antibody **(figure-7)**. This is in accordance with the findings of **Dwjvestijn et al. (1992, p.459)**, in which it was found that the RECA-1 was endothelial cell- specific whereas all other antibodies cross-reacted with one or more other cell types. No reactivity of RECA-1 was found in various tested species other than rat. In this study, the RECA-1 antibody was successfully applied in staining of paraformaldehyde fixed, plastic embedded tissue material. Immunofluorescence staining of viable endothelial cells demonstrated that RECA-1 recognizes a cell surface antigen. This was supported by intravenous injection of RECA-1, which showed the antibody to localize along the endothelium lining the vasculature in various organs tested.

Glucose transporters (GLUTs) at the blood-brain barrier maintain the continuous high glucose and energy demands of brain. They also act as therapeutic targets and provide routes of entry for drug delivery to the brain and central nervous system for treatment of neurological and neurovascular conditions and brain tumors **(Patching, S. G. 2017, p. 1046).**On performing the immunohistochemistry for GLUT 1 in frozen sections of hypothalami from normal rats, GLUT 1 was present on the endothelial cells of the blood brain barrier (BBB) **(Nagarmukos et.al. 2001,p.1).**Similar findings are present in this study and no change of immunoreactivity is seen in treatment groups as compared to control group **(figure-8)**.

CD34 is a transmembrane highly glycosylated protein, which has been extensively used as a marker of hematopoietic progenitor cells and non-hematopoietic progenitor cells. CD34 can regulate trafficking and migration of hematopoietic progenitor cells, which eventually may migrate to the CNS and differentiate into microglia **(Kovacs et al., 2019, p.2)**. In the present study, in all the groups, the endothelium of blood vessels and capillaries in the brain parenchyma show complete and continuous membranous moderate intensity of immunoreactivity in most of blood vessels **(figure-9)** whereas it was found by **Kovacs et al., (2019, p.2)** that following CNS damage, CD-34 expressing microglia have been found in affected regions, which are also characterized by microgliosis and blood brain barrier damage.

Glial cells in the central nervous system (CNS) maintain the homeostasis of the internal environment. Glial cells and especially astrocytes (AS) form glial scars through activation and proliferation. AS secrete a variety of inhibitory factors stimulated by injury, ischemia, blood brain barrier damage, inflammatory reaction, abnormal metabolism and oxidative stress. These external factors critically affect axon regeneration and recovery of neurological function in the CNS. Increased expression of glial fibrillary acidic protein (GFAP) is one of the most important markers of AS activation **(Zhang et al.2017, p.1905)**.

In the present study, in all the groups while investigating the antibody expression in different areas of rat brain, the immunoreactivity of end foot processes of astrocyte cells around most of the blood vessels and capillaries show moderate intensity and complete continuous immunoreactivity **(figure-10)**.

The studies show that GFAP inhibition increased neuronal death, suggesting the protective effects of GFAP in the nervous system injury, the AS intermediate filament protein system, which includes GFAP, is not only an important component of the cytoskeleton but also a vital signaling system that responds to a variety of stress conditions, and regulates cell size, migration and survival via multiple proteins**(Zhang et al.2017, p.1905).**In the present study there was no change in the immunoreactivity in all the treatment groups as compared to control group after administration of Abhrak bhasm drug.

S100 exists in wide variety of tissues and cell types, originally isolated from brain tissue and they are of low molecular weight proteins. S100 is evenly distributed in cytoplasm and also in nucleoplasm and is involved in both intracellular and extracellular functions.S100 protein is generally expressed in normal and also in pathological conditions **(Kuberappa et al.2016, p.10)**. In the present study there was no change in the expression of S100 antibody in different regions of the brain in all the groups and S100 showed continuous moderate intensity of immunoreactivity around the blood vessels and capillaries **(figure-11)**.

The studies conducted by **Bilinov & Terent'ev, (2013, p.169)** showed that Immunohistochemical studies on the antigenic composition of the brain tissue over a 20 – year period have made it possible to describe more than 120 different NSPs. Glial fibrillary acidic protein (GFAP), a structural protein of astrocytic intermediate filaments, and the S100 family of proteins are localized in astrocytes and are widely used in experimental and clinical studies of the BBB.

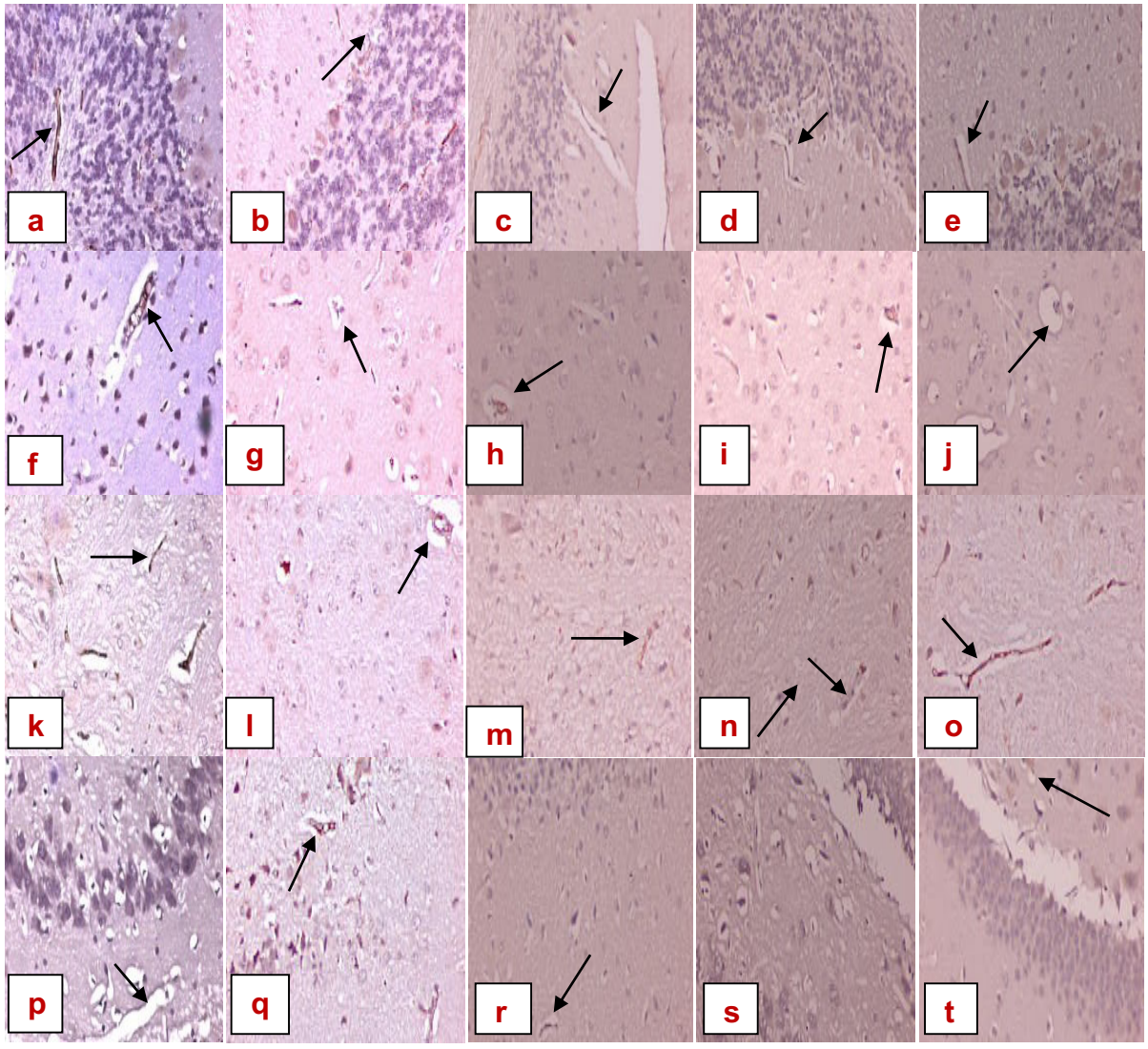

Fig.6- Occludin antibody expression in different areas of rat brain. Fig. a, f, k & p show cerebellum, cerebral cortex(frontal f, occipital k) and hippocampus of control group respectively and blood vessels, capillaries show more intensity of immunoreactivity as compared to rest of the groups. Fig. b, g, I &q show cerebellum, cortex (frontal j,occipital i) and hippocampus of group A respectively and the intensity of blood vessels of occipital region is more than frontal region. Fig.c,h,m & r show cerebellum, cortex (frontal h, occipital m) and hippocampus of group B rat respectively. Fig.d, i, n & s show cerebellum, cortex (frontal I, occipital n) and hippocampus of group C rat respectively. Fig. e, j, o & t show cerebellum, cortex (frontal j, occipital o) and hippocampus of Group D rats respectively and blood vessels of group A,B,C&D show similar intensity with each other but weaker than control group. **(as shown by arrows).**

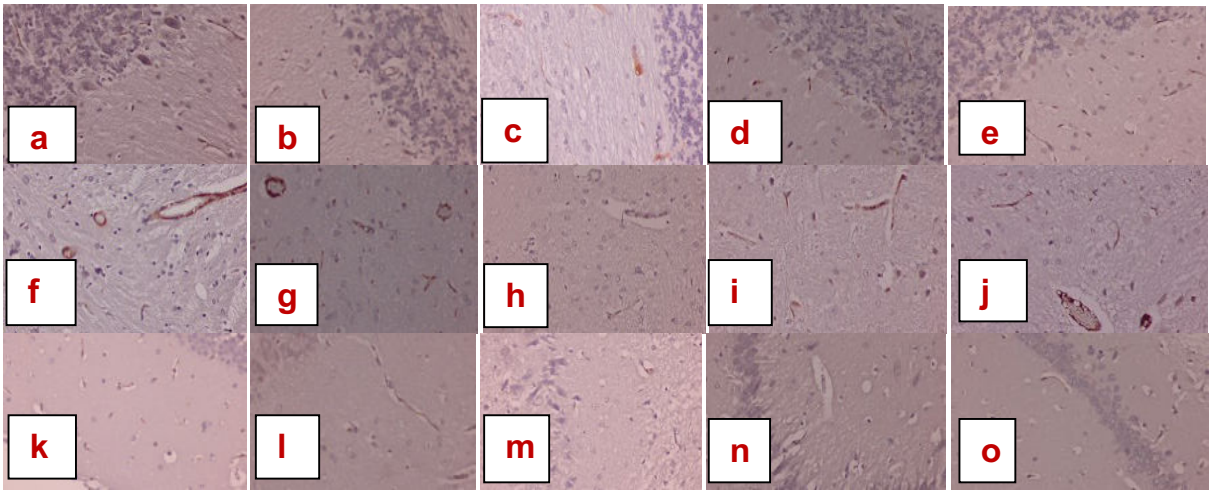

Fig.7- RECA 1 antibody expression in different areas of rat brain. In Control group, blood vessels show strong intensity of immunoreativity in cerebellum, cortex and hippocampus regions in figures. a, f& k respectively. Similar findings were seen in group A (figures b, g & l); in group B (figures c, h & m); in group C (figures d, i and n) and in group D (figures e, j & o) respectively. There is no change in immunoreactivity in treatment groups as compared to control group.

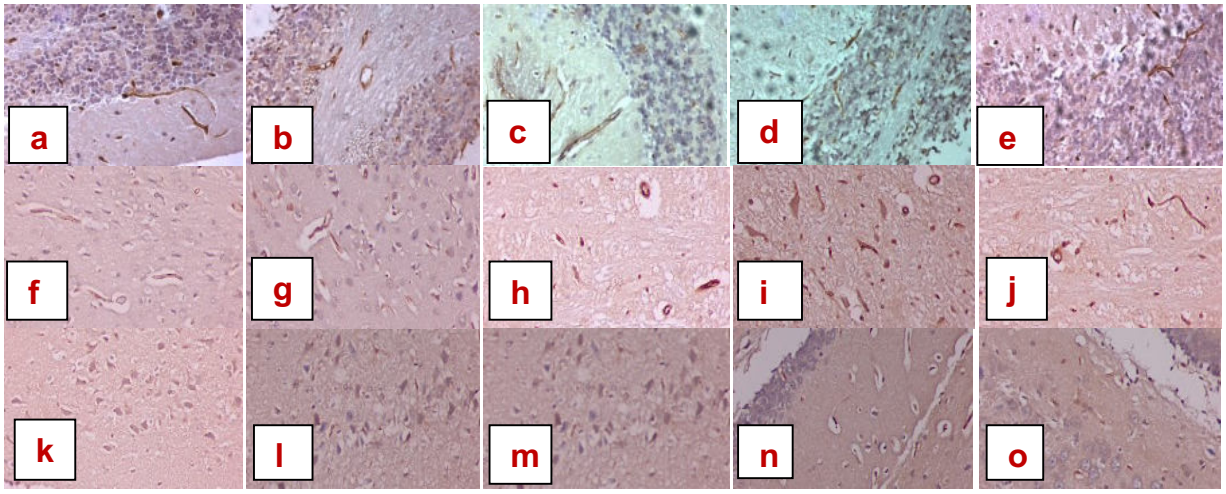

Fig.8- GLUT 1 antibody expression in different areas of rat brain. In Control group, blood vessels show strong intensity of immunoreativity in cerebellum, cortex and hippocampus regions in figures. a, f& k respectively. Similar findings were seen in group A (figures b, g & l); in group B (figures c, h & m); in group C (figures d, i and n) and in group D (figures e, j & o) respectively. There is no change in immunoreactivity in treatment groups as compared to control group.

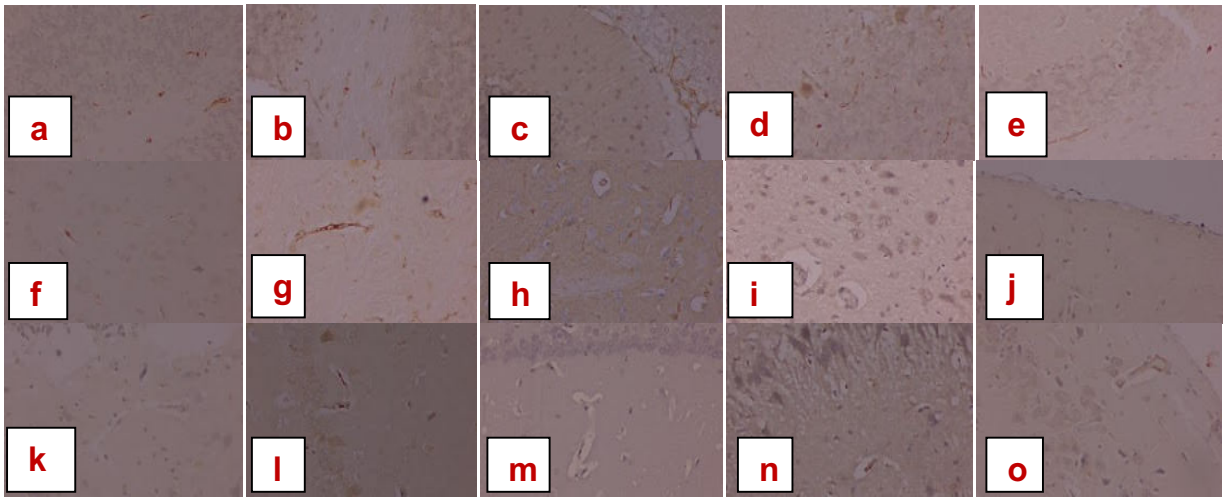

Fig.9- CD 34 antibody expression in different areas of rat brain viz. blood vessels of cerebellum, cortex and hippocampus region of rat brain in Control group (Figures- a,f&k); in group A (Figures- b,g & l); in group B (Figures-c,h&m);in group C (Figures-d,i &n); in group D (Figures-e, j & o). In all the groups, the endothelium of blood vessels and capillaries in the brain parenchyma show complete and continuous membranous moderate intensity of immunoreactivity in most of blood vessels.

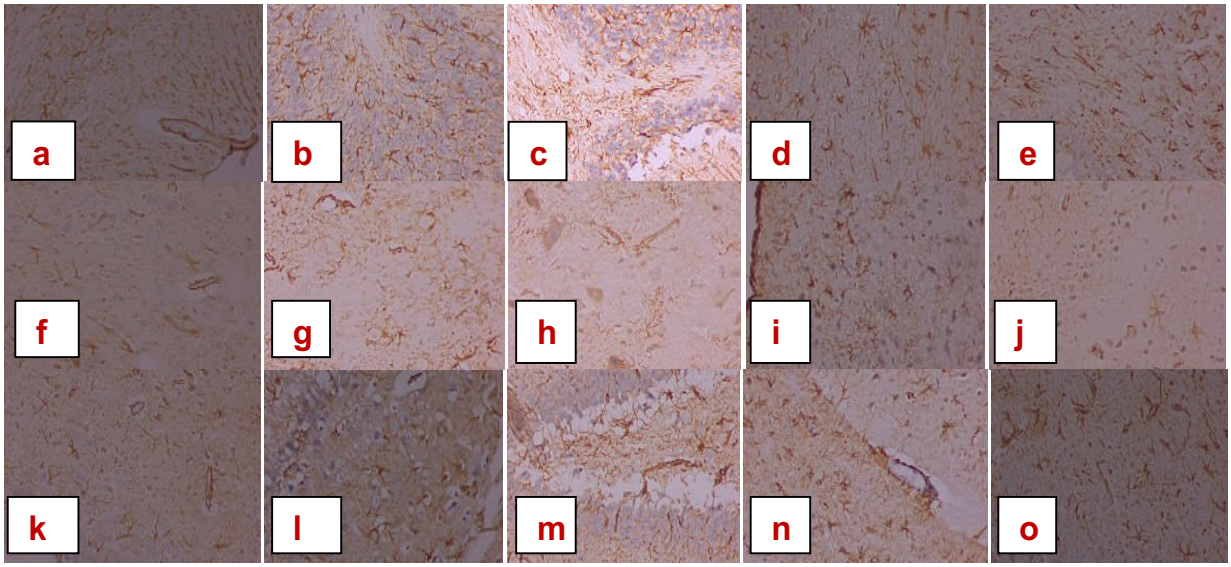

Fig.10- GFAP antibody expression in different areas of rat brain viz. blood vessels of cerebellum, cortex and hippocampus region of rat brain in Control group (Figures- a,f&k); in group A (Figures- b,g & l); in group B (Figures-c,h&m);in group C (Figures-d,i &n); in group D (Figures-e, j & o).In all the groups , the immunoreactivity of end foot processes of astrocyte cells around most of the blood vessels and capillaries show moderate intensity and complete continuous immunoreactivity

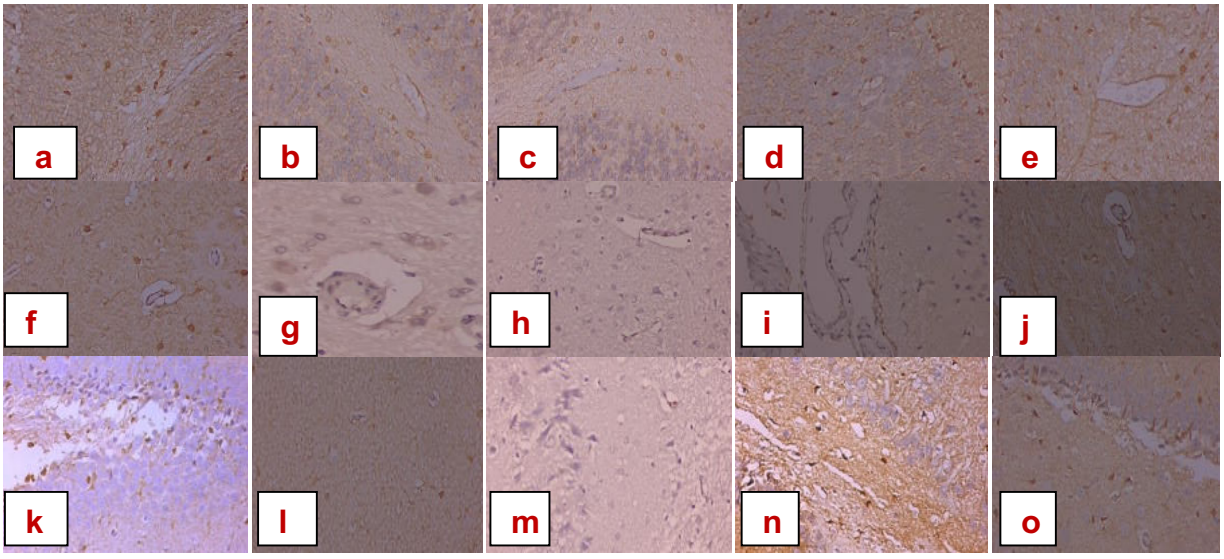

Fig.11- S100 antibody expression in different areas of rat brain viz. blood vessels of cerebellum, cortex and hippocampus region of rat brain in Control group (Figures- a,f&k); in group A (Figures- b,g & l); in group B (Figures-c,h&m);in group C (Figures-d,i &n); in group D (Figures-e, j & o). In all the groups S 100 show continuous moderate intensity of immunoreactivity around the blood vessels and capillaries.

Conclusion:- It is a novel study in which immunohistochemistry of blood brain barrier is done by using six research markers, specific to the cells of blood brain barrier, after administration of Abhrak bhasma in rats. On the basis of findings of present study it can be concluded that the therapeutic dose of Abhrak bhasma causes changes at the level of tight junctions present in blood brain barrier in rats which is shown by immunohistochemistry with occludin research marker i.e. a tight junction protein marker. There is no toxic effect of drug on different organs of rats as no significant changes in histology of organs are seen. More studies need to be done to check the permeability of blood brain barrier for Abhrak bhasma drug, like calculating its concentration in brain tissues and other vital organs of rat so that it may be determined that it may enter the brain tissue after crossing the blood brain barrier and also to see its effectiveness in different CNS disorders.

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Conflict of Interest:- None

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