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Hypothyroidism Alters Cell Death/Inflammatory Pathways In A Rat Model Of Transient Focal Cerebral Ischemia

[*1]Dr. Leena Rastogi
Msc, Phd
Department Of Zoology
Baba Saheb Bhim Rao Ambedkar University,Vidya Vihar
Lucknow-226025

[2]Dr. Vanshika Rastogi
MBBS,MD
Department Of Radiation Oncology, Sanjay Gandhi Post Graduate Institute Of Medical Sciences, Raebareli Road, Lucknow-226014

Abstract
Clinical and experimental observations suggest that a high level of thyroid hormone aggravates injury whereas low levels offer protection in stroke patients and in animal models. The mechanism of neuroprotection by altering cell survival or death pathways by hypothyroidism remains unclear. In this study, induction of transient focal ischemia has been done by occlusion of right middle cerebral artery for 2 hrs followed by 1 and 3 days of reperfusion in hypothyroid and euthyroid rats. Decrease in neurological deficit score, infarct volume, pro-apoptotic/inflammatory proteins and upregulation in antiapoptotic proteins was observed in the brain of hypothyroid ischemic reperfused rats. Collectively, these results underscore the mechanistic basis of low thyroid hormone status as a protective factor in acute stroke.

Keywords: *Apoptosis, Thyroid Hormone, Transient focal ischemia, Rat, Necrosis*

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I. Introduction

Cerebral ischemia affects multiple physiological targets and biochemical pathways resulting in morbid and very often fatal consequences. Cell death, including necrosis and apoptosis contribute to the final morbid consequences (Liu et al., 2018; Radak et al., 2017). It has been seen the protective role of pre-existing hypothyroidism on outcome of cerebral ischemia in gerbils, rats, and humans (Akhoundi et al., 2011; Alevizaki et al., 2006, Rastogi et al., 2006; Shuaib et al 1994). Hypothyroidism induced neuroprotection during ischemic injury involves down regulation of glutamate release and attenuation of ROS, calcium levels, release of LDH from brain tissue (Lee et al., 2010; Rastogi et al., 2008, 2018). Both oxidative stress and calcium elevation has been known to cause mitochondrial dysfunction and initiation of various inflammatory response in the damaged neurons (Lemasters et al., 1998; Maddahi et al., 2011; Ouyang et al.,1999). Underlying mechanism on these pathways of neuronal damage during Ischemic injury is still not clear. The effect of hypothyroidism on mitochondrial and inflammation mediated signalling during ischemia reperfusion is studied. Though establishment of hypothyroidism is desirable but not feasible in clinics, but molecules involved in protection can be used as therapeutic targets. The molecular mechanisms controlling potential harmful stress responses and preserving cytoprotective responses under hypothyroid condition is not clear.

II. Materials and Methods:

Chemicals

Radioimmunoassay kits for the determination of thyroid hormone levels were obtained from Diagnostic Products Corporation Los Angeles, CA. All other chemicals were purchased from Sigma Chemical Company, St Louis, MO, USA and Sisco Research Laboratory, India.

Antibodies

Antibodies against TNF-α (52B83) sc-52746; β-actin (ACTBD11B7) sc-81178; AIF(E-1) sc-13116; BCL-2(C-2 sc-7382) [Santa Cruz Biotechnology, Santa Cruz, CA], cleaved Caspase-3 (Asp175) #9661; BAX (D3R2M) #9661; COX IV (4D11-B3-E8) #11967 [Cell Signalling Technology, Beverly, MA], NOS2 (SAB4502011) [Sigma-Aldrich, USA] were purchased. Cytochrome c (7H8.2C12; Dr. R. Jemmerson), NF-kB/p50 (Dr. M.K. Ernst) were of generous gift. Secondary biotinylated antibody, streptavidin–HRP conjugated and Diaminobenzidine were used from Vecta Stain Universal Quick Kit (Vector Laboratories).

Animals

Adult rats (Sprague Dawley, weight 225-250 g) were taken for this study. Rats were maintained on 12-hr light-dark cycle in optimum temperature with food and water (ad libitum). Two group of rats are formed: Euthyroid (E) & Hypothyroid (H). Hypothyroidism was induced by giving methylimidazole (MMZ, 0.025% w/v, 6 weeks) in their drinking water & Thyroid Hormone levels were measured in blood samples of rats by kit (Diagnostic Product Company, New York, USA). Experimental animal procedures of the study were done in accordance with the guidelines of Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Sham operated Euthyroid (n=15) & Hypothyroid (n=15) control group rats designated as "C" and rats who underwent ischemia/reperfusion for 1 and 3 days were labelled as EIR-1(n=15), EIR-3 (n=15) and HIR-1(n=15), HIR-3 (n=15) respectively. Body weights & temperature of rats were taken regularly. Blood pressure and heart beat of animals were recorded by polygraph, (Grass Instruments Co., USA).

Induction of focal ischemia/reperfusion in rats

Rats from both the groups were fasted overnight, anaesthetized (Chloral hydrate 300mg/kg, ip) and induction of focal ischemia/reperfusion was done by inserting nylon suture up to origin of right middle cerebral artery (MCA) for 2 hr (Zea Longa et al., 1989) & retracting the nylon suture (reperfusion) for 1 & 3 days. The sham operation was performed in euthyroid and hypothyroid rats without occluding the right middle cerebral artery. Body temperature 36-38°C was maintained during and after surgery by using a heating lamp.

Blood and brain tissue collection:

Blood was collected from each rat under anaesthesia. Transcardial paraformaldehyde perfusion for preparation of paraffin embedded brain section and PBS perfusion for fresh brain tissue was performed prior to dissection.

Right hemisphere (ipsilateral) tissue was used for preparation of mitochondrial and post mitochondrial fractions. Immunohistochemistry and immunofluorescence were performed in paraffin embedded brain sections.

Neurological/ Behavioural evaluation

Neurological evaluation was done in rats by using a 6-point postural reflex test (Bederson et al., 1986) daily, up to 3 days of reperfusion.

Infarct Assessment & quantitation

Infarct assessment was done by staining of fresh brain tissue with TTC (2,3,5 -triphenyl tetrazolium chloride). Seven sections of brain were cut and incubated in 2% solution of TTC for 30 mins. The infarction area of each brain section was quantified using image analysis system software (Image plus, Expert Vision Labs Pvt. Ltd., India). Quantitation of infarct volume was done by addition of the infarct areas of seven sections and multiplying it by slice thickness (2 mm).

Western Blotting

Brain tissue (Ipsilateral side) from all group of rats were taken, homogenised and differential centrifugation has been done by standard protocol to obtain mitochondria (resuspended in Tris-HCl buffer; mitochondrial fraction) and supernatant (post mitochondrial fraction/ cytosol) & stored at -80° C until use for protein expression analysis. Post mitochondrial fraction was used for analysis of Cytochrome c, AIF, NOS2, TNF-α, BCL2 and β actin whereas mitochondrial fraction was used for BAX, and COX IV analysis.

50µg protein from the mitochondria and post mitochondrial fractions were separated by SDS- PAGE (6, 12-15% gel according to weight of protein) electrophoresis with molecular weight marker. After electrophoresis, the protein was electro transferred from gel to nitrocellulose membrane. The non-specific blocking in the membrane was done with non-fat milk for 1hr at 37°C and incubated with different primary antibodies (dilution Cytochrome c 1: 2000, AIF 1: 1000, BCL2 1:100, BAX 1:1000, NOS 2 1:1500, TNF-α 1:1000) for overnight at 4° C. β-actin (post mitochondrial fraction) and COX-IV (mitochondrial fraction) were used as control for protein expression analysis. Primary antibodies were detected by corresponding horseradish

peroxidase (HRP)- conjugated secondary antibodies (anti-mouse 1:2000 for Cytochrome c, anti-goat 1:500 for AIF, anti-mouse 1:1000 for BCL2, anti-rabbit 1:1000 for BAX, anti-rabbit NOS2 1:1000 & anti-mouse TNF-α 1:1000) using enhanced chemiluminescence substrate (GE Healthcare, USA). Densitometric analysis was done using Lab work 4 (Ultra-Violet Products Ltd, Cambridge, UK) for quantitation.

Immunohistochemistry:

Paraffin embedded brain sections (4µm) (Leica Biosystem RM2245 Semi automated Rotatory microtome) were deparaffinized at 56^{0} C, rehydrated and subjected to antigen retrieval in 10 mM sodium citrate buffer (pH 6.0).The blocking of the nonspecific binding in brain sections were done with 10% normal sheep serum for 1 hr at 37°C followed by incubation with primary antibody of NOS2 (1:1000 dilution) for 2 hr at 37°C.The sections were incubated with biotinylated universal secondary antibody for 20 min after rinsing the slides with PBS three times. Sections were incubated with streptavidin–HRP conjugated complex for 10 min. Diaminobenzidine [DAB] was used as chromogen and hematoxylin as counterstain for sections. Counting of DAB positive cells in brain sections was done with Image J Fiji (software version 2) to all groups of rats.

Immunofluorescence:

Expression of cleaved Caspase-3 and NF-kB were done in paraffin embedded brain sections (4µm) separately. After deparaffinization, rehydration and antigen retrieval in brain sections of all group of rats, sections were treated with 10% normal sheep serum for 1 hr to block the nonspecific binding. The sections were incubated with primary antibodies of rabbit polyclonal cleaved-caspase-3 (1:500; Cell Signaling Technology), and rabbit polyclonal NF-kB/p50 (1:200) antibody overnight at 4^{0} C separately. Sections were incubated with Rhodamine Red-conjugated anti- rabbit IgG secondary antibody for cleaved Caspase-3 and NF-kB for 1 hr after washing with PBS three times. Sections were counterstained with Hoechst-33258 (0.5µg; a nuclear dye) and visualized under a fluorescent microscope (Olympus).

Protein concentration was assayed in mitochondrial and post mitochondrial fractions (Bradford M.M.,1976) using BSA as standard.

Statistical significance

Statistics for quantitative variables are presented in mean ±SD whereas categorical variables in frequency and %. Analysis of variance test (one way) was applied, & multiple comparison using SNK test were done among groups. Similarly, independent sample *t*- test were used when comparing between two groups. Fischer's exact test was applied to compare the proportion between the groups as appropriate. Statistical Package for Social Sciences version 23 (SPSS 23, IBM, Chicago, USA) was used for data analysis & statistical significance. A p-value <0.05 was considered significant.

III. Results

MMZ treatment had produced a hypothyroid state in rats (Table 1). At the beginning of experiment before occlusion, different groups of rats showed normal behavioural reflex (score=0). Rats in EIR-1, EIR-3, HIR-1 and HIR-3 group showed different neurological deficit on scale of 0 to 5. 66% (10/15) rats in EIR-1 group showed deficit on the scale of 1-2 and 33% (5/15) on the scale of 3-4, whereas 60% (9/15) rats in EIR3 group showed more deficit of scale 3-4 and 40% (6/15) showed score of 1-2. In HIR-1 and HIR-3 rats, only 20% (3/15) rats showed deficit score of 1-2, 80% (12/15) rats showed postural reflexes (score= 0, Table 2) respectively.

TTC stained brain sections were indicating unstained area as brain tissue infarct (Fig. 1A). Total infarct area in ipsilateral side of EIR-1 was 95±20 $mm^{2,}$ ($p<0.05$), EIR-3 rats was 145±20 mm^2 ($p<0.01$), whereas infarct area in HIR-1 was 55±15 mm^2 ($p<0.05$) & HIR-3 rats was 70±10 mm^2 ($p<0.05$).

Similarly, significantly larger infarct volume was observed in EIR-1 (190 ± 32.5 mm^3) and EIR-3 rats (290 ± 29.0 mm^3) in comparison with sham operated control group C ($p < 0.01$). HIR-1, HIR-3 rats showed infarct volume of 100 ± 20.6 mm^3; 140 ± 25.5 mm^3 respectively ($p<0.01$) (Fig. 1 B).

Hypothyroidism reduces intrinsic mitochondrial apoptotic signalling during IR injury in rats

Cytochrome c immunoreactivity was mildly detected in the sham operated C rats from both the groups. Expression of Cytochrome C which increased 2 folds in EIR-1, 3 groups ($p< 0.05$) whereas levels were unaltered in HIR1 and HIR-3 (Fig.2A,2B).

Expression of AIF was increased in EIR-1 and EIR-3 rats (2A, 2B) and densitometry analysis demonstrated significant 3-fold increase in immunoreactivity in EIR-1 and EIR-3 rats ($p<0.01$) when compared to euthyroid C, AIF levels were not remarkedly increased in HIR-1 and HIR-3 groups. Expression of BCL2 protein was observed in all group of rats. Notably, increased expression of BCL-2 protein was observed in HIR-1 and HIR-3 ($p<0.05$) whereas in EIR-1 and EIR -3 groups slight decreased expression was observed (Fig. 2E,

2F). Significant increased expression in pro-apoptotic protein BAX was observed in EIR-1 ($p<0.05$), whereas expression of BAX protein was unaltered in H, HIR-1 and HIR-3 (Fig. 2E ,2F).

Hypothyroidism reduces pro inflammatory signalling during IR injury in rats

TNF- α immunoreactivity was mildly detected in sham control groups. TNF alpha levels were increased in 1.5 and 3 folds in EIR-1 and EIR-3 groups ($p<0.05$) whereas TNF alpha unaltered in HIR-1 and HIR-3 groups (Fig. 2C,2D).

Densitometric analysis of western blot showed significant 5- & 6-folds increased expression of NOS2 in EIR-1, EIR-3 groups respectively ($p<0.01$) whereas NOS2 expression was not observed in H, HIR-1, HIR-3 groups (Fig. 3A, 3B). Immunohistochemical analysis showed increased % of Diaminobenzidine (DAB) positive cells in EIR-1 and EIR-3 groups when compared with E group. Few DAB positive cells were observed in HIR-1, HIR-3 (Fig 3 C, 3D) when compared with respective control.

Immunofluorescence studies reveals transcription factor NF-kB was translocated from cytoplasm to nucleus in EIR-1, EIR-3 ($p<0.01$) (Fig.4 A,4B). Significant reduction in NF-kB translocation in nucleus was observed in HIR-1 and HIR-3 groups ($p<0.05$). Cleaved caspase-3 in the nucleus of EIR-1 and EIR-3 was observed (5A) and increased was 8 and 15 folds respectively (5B) ($p<0.01$)

IV. Discussion:

Stroke is multifactorial disease condition that is represented by ischemia of the brain due to decrease blood supply. Oxidative stress has crucial role in augmenting the ischemic reperfusion injury causing cell death by generation of reactive oxygen species, induced Calcium levels, excitotoxicity, and inflammation (Lemasters et al., 1998; Lee et al., 2010; Maddahi et al., 2011; Ouyang et al., 1999; Rastogi et al., 2008; 2018).

Previously we have shown that hypothyroidism protect the rat brain from ill effect of ischemia-reperfusion by decreasing oxidative stress, necrosis (Rastogi et al., 2006). Further, protective effects of hypothyroidism were associated with an inhibition of apoptotic and inflammatory events in the brain along with induction of antiapoptotic protein BCL-2 on brain injury in the current study.

Significant increased expression of TNF -α & nuclear localization of NF-kB in EIR-1 and EIR-3 group of rats when compared to E control ($p<0.05$). Deleterious cytokine TNF-α, was demonstrated to mediate inflammatory & vascular changes and facilitates NF-kB translocation to nucleus initiating a vicious cycle leading to further generation of free radicals and apoptotic event (Bohlers et al., 2000, Clemens et al., 1999; Duckworth et al., 2006; Hu et al., 1997; Schulz et al., 1999). NOS2 is inducible form and expressed after brain injury (Bonfoco et al., 1995). Western blot & Immunohistochemical analysis in brain sections of EIR-1 and EIR-3 showed increased expression and number of NOS2 positive cells. NOS2 enzyme is responsible for increased synthesis of Nitric oxide, a reactive free radical which interact with OH radicals, forms more reactive peroxynitrite which has deleterious effect on cell biomolecules and act as a trigger for ischemic cell death signalling (Bonfoco et al.,1995). Mitochondrial dysfunction is implicated in both apoptotic and necrotic cell death through ROS (Alevizaki et al 2006). Release of Cytochrome c & AIF from mitochondria into the cytoplasm accompanied by loss of mitochondrial membrane potential was seen in ischemia reperfusion injury (Lemasters et al., 1998) and we also observed the significant increase in Cytochrome c and AIF in EIR1 and EIR3 rats.

BAX is a mediator of mitochondrial dependent apoptotic cell death, whose activity is neutralized by binding with BCL2 (Chen et al., 1995; Wu et al., 2003). In our results, low expression of the BCL2 and high expression of BAX were found in EIR rat brain in association with increased cytochrome c and AIF release. Interestingly, increased expression of BCL2, and decreased levels of BAX with decreased release of Cytochrome C, AIF which protects hypothyroid rat brain from ill effect of ischemia reperfusion injury.

In hypothyroid ischemic reperfused rat expression of TNF alpha is significantly decreased in comparison to euthyroid ischemic reperfused rat. It is suggested that decreased TNF-α level, no expression of NOS2, decreased translocation of NF-kB inhibit apoptosis in hypothyroid rat brain after injury. ROS is implicated in the process of apoptosis, and mediate cytokine (TNF-α, and IL-1α) - induced apoptosis (Bonfoco et al., 1995)

Enhanced BCL2 expression was observed in HIR1 and HIR3 rats when compared with EIR-1and EIR-3 rats respectively ($p<0.05$). A significant role for BCL2 in promoting cell survival and cell death was reported (Chen et al., 1995). BCL2 protein has been shown as inhibitor of apoptosis. BCL2 protects the integrity of mitochondria and thus limits mitochondrial dysfunction induced by several apoptosis stimuli (Wu et al., 2003; Zhao et al., 2003).

The loss of mitochondrial membrane potential & release of Cytochrome c and AIF from mitochondria are critical step to initiate the apoptotic cell death pathways (Pradeep et al., 2012). The effector molecule in apoptosis cascade is activation of caspase-3 which acts on the nucleus and DNA is fragmented. Anti-apoptotic BCL-2 resides in mitochondrial membrane and block release Cytochrome c and AIF from mitochondria thus

inhibiting activation of caspases in the cytoplasm (Zhao et al., 2003). NF-kB is activated in response to several stimuli, most of which represent pathological stresses such as ischemia reperfusion or by cytokines. Activity of NF-kB is down regulated by antiapoptotic protein BCL2. Ischemic neuronal death involves a spectrum of necrotic and apoptotic mechanisms (Fulda et al., 2010; Talhada et al., 2019; Nikoletopoulou et al., 2013).

We observed less activation of Caspase-3 and low release of Cytochrome c in HIR rats in this study. There is also increased BCL2 expression in HIR rats compared to EIR rats. It is reported that BCL2 allows cells to adapt to an increased state of oxidative stress by suppressing the apoptosis, either by neutralizing the free radicals or by enhancing the cellular antioxidant defences (Duckworth et al., 2006).

There is low expression of apoptosis related proteins in HIR rats. Hypothyroidism limits infarct size and 80% rats showed normal postural reflexes after injury, suggesting low levels of thyroid hormone have protective effect on functional outcome. Thus, our results support the fact that hypothyroidism protects the brain from ill effects of ischemia/reperfusion by modulating anti apoptotic and apoptotic molecules. In concurrence with earlier reports (Akhoundi et al., 2011; Lee et al., 2010; Rastogi et al., 2006) that hypothyroidism attenuates oxidative stress and hence necrosis in ischemic reperfused rats, we may conclude from our study that low levels of thyroid hormone attenuate the sequence of necro-apoptosis and is neuroprotective.

The only effective modality is tissue Plasminogen Activator (t-PA) given within 3 hrs of onset of ischemic stroke in patients. Our present observations and previous study in animals demonstrating a protective role of reverse T_3 in experimental ischemic brain injury in animal models may have potential applications.

Further research needs to focus on methods of rapidly reducing the levels of thyroid hormones selectively on the brain in relation to ischemic stroke in human beings and the potential utility of this approach in stroke therapy.

Conflict of Interest: There is no conflict of interest between authors

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Table 1

Bodily functional parameters in Euthyroid and Hypothyroid rats

Groups	Body weight (gm)	Blood Pressure (mm Hg)	Heart Rate (beats/min)	Thyroid hormone T_3 (nM) T_4 (nM)	Body temp in ^oC
Euthyroid	250±15	110±8	270±15	1.3±0.02 55±10	37±1
Hypothyroid	230±10	90±5*	230±10	0.80±0.04* 10±1*	36±1

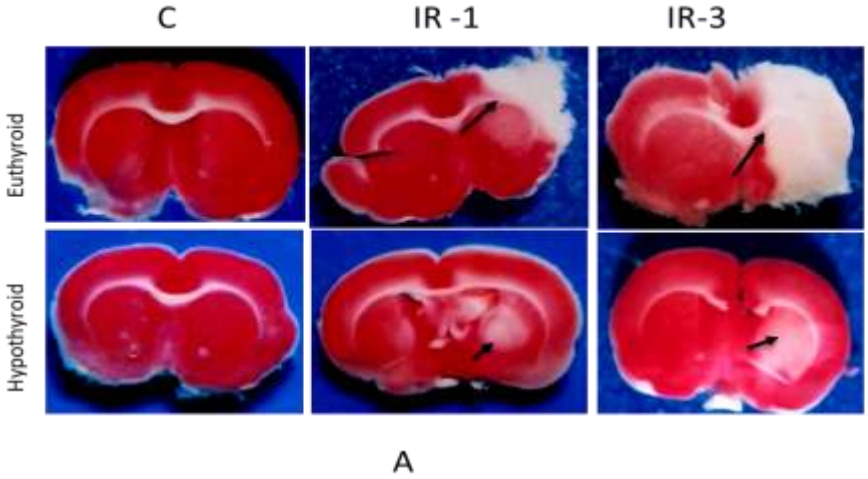
Blood pressure, heart rate was recorded in animals of the both group (n=5) and other parameters were estimated in animals of both the group (n=15). Values were mean ± SD and statistical significance was assessed by Student's t test. * $p< 0.05$ when compared between euthyroid and hypothyroid rat.

Table 2:

Neurological deficit score in Euthyroid and Hypothyroid rats (sham C, IR-1, IR-3)

Neurological deficit score	Groups			
	EIR-1 (n=15)	EIR-3(n=15)	HIR-1(n=15)	HIR-3 (n=15)
0	0	0	12 (80%)	12 (80%)
1-2	10 (66%)	6 (40%)	3 (20%)	3 (20%)
3-4	5 (33%)	9 (60%)	0	0

n=15 rats were taken in each group for assessments of neurological deficit score (scale from 0-5). Sham-operated rats from both groups show normal reflexes (score 0). The number of rats in different groups was shown in the table with neurological deficit scores. In parenthesis, the percent of rats were shown against the score. Fisher's exact test to compare the proportion between the groups.



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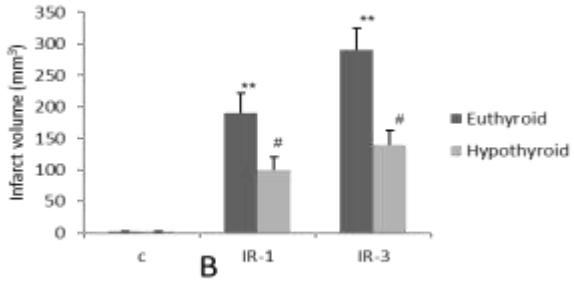

B

Figure 1 . Hypothyroidism protects rats against ischemia-reperfusion injury. (Fig.1A) Infarct assessment in different groups of rats by TTC staining on brain sections. Non-stained area of the brain section shows the infarct in brain tissue. The decrease in infarct volume in HIR-1 and HIR-3 was significant (Fig.1B). Values are mean ± SD (n=5), **p<0.01 vs "C", #p<0.05 vs respective IR.

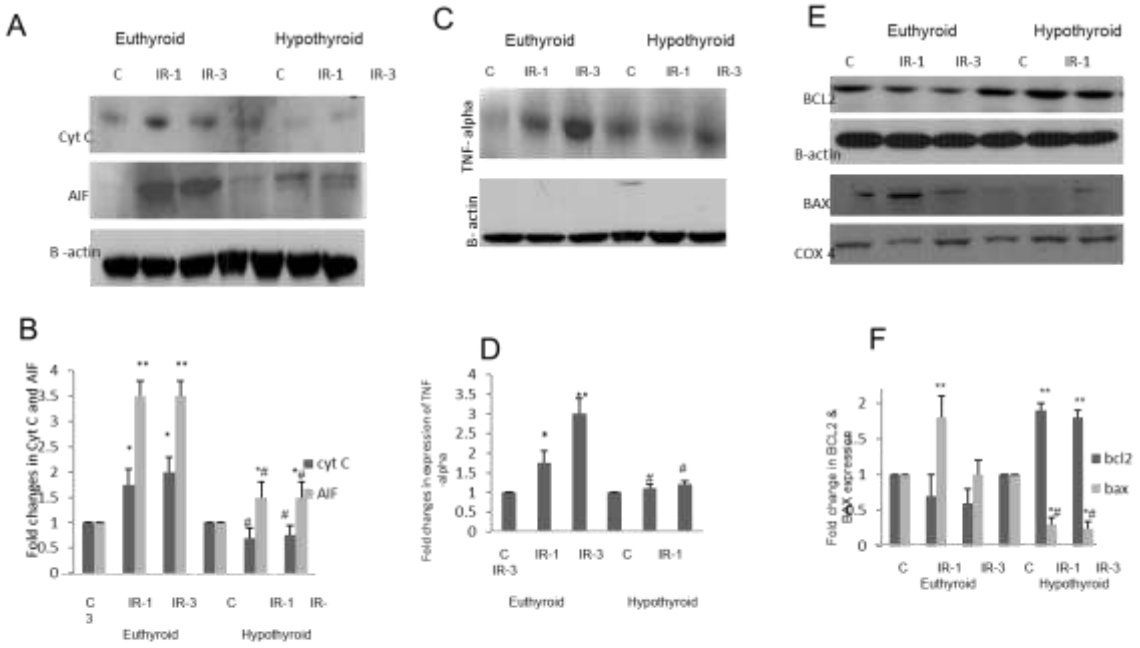

Figure. 2: Immunoblots (A, C, E) & densitometric (B, D, F) analysis of Apoptogenic and Anti apoptogenic proteins in brain tissue of different groups of rats. A, Cytochrome C; B, Apoptosis Inducing Factor (AIF); C & D TNF-α ; E , BCL2 in post mitochondrial fraction ; F, BAX in mitochondrial fraction of brain tissue. A, Representative blots showing the effects of ischemia reperfusion on Cytochrome c and AIF expression in cytoplasm of euthyroid and hypothyroid rats. B, Fold changes in density of Cytochrome c and AIF in euthyroid and hypothyroid group relative to their respective control group. C, D TNF -α is down regulated in hypothyroid ischemic rat brain. E, F significant increase in BAX proapoptotic protein in IR-3 euthyroid group of rats. Significant reduction in BAX protein in hypothyroid ischemia reperfusion rats when compared to respective euthyroid ischemia reperfusion rats. β-actin & COX IV were used as a control for cytosol & mitochondria respectively. Each group represents the mean ± SD of five animals. *p<0.05, **p< 0.01 versus control; #p < 0.05 versus respective I R.

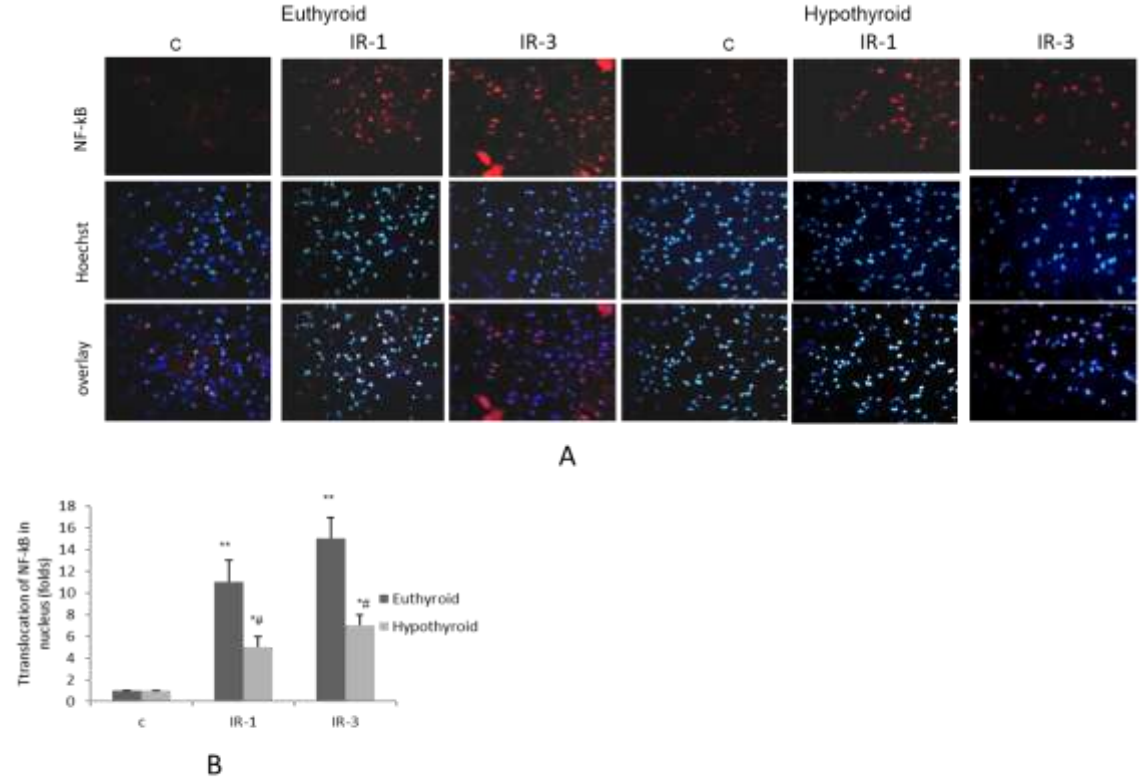

Figure 4: Paraffin embedded tissue sections were prepared from euthyroid & hypothyroid control (C) as well as ischemia reperfusion for 1 day (IR-1) and 3 days (IR-3). (A), Immunofluorescence for NF-kB in MCA territory of ischemic cortex (red). Brain section were stained with nuclear dye Hoechst (blue) and NF-kB (red). In the merged figures (overlay panel), pink colour indicates localization of NF-kB in the nuclei. (B), change in number of NF-kB translocated from cytoplasm to nucleus in euthyroid and hypothyroid- ischemic reperfused rats relative to sham operated control. Bars represent means of number of cells having nuclear localization of NF-kB ±SD. *p<0.05, p**<0.01 vs sham operated control, #p < 0.01 versus respective IR.

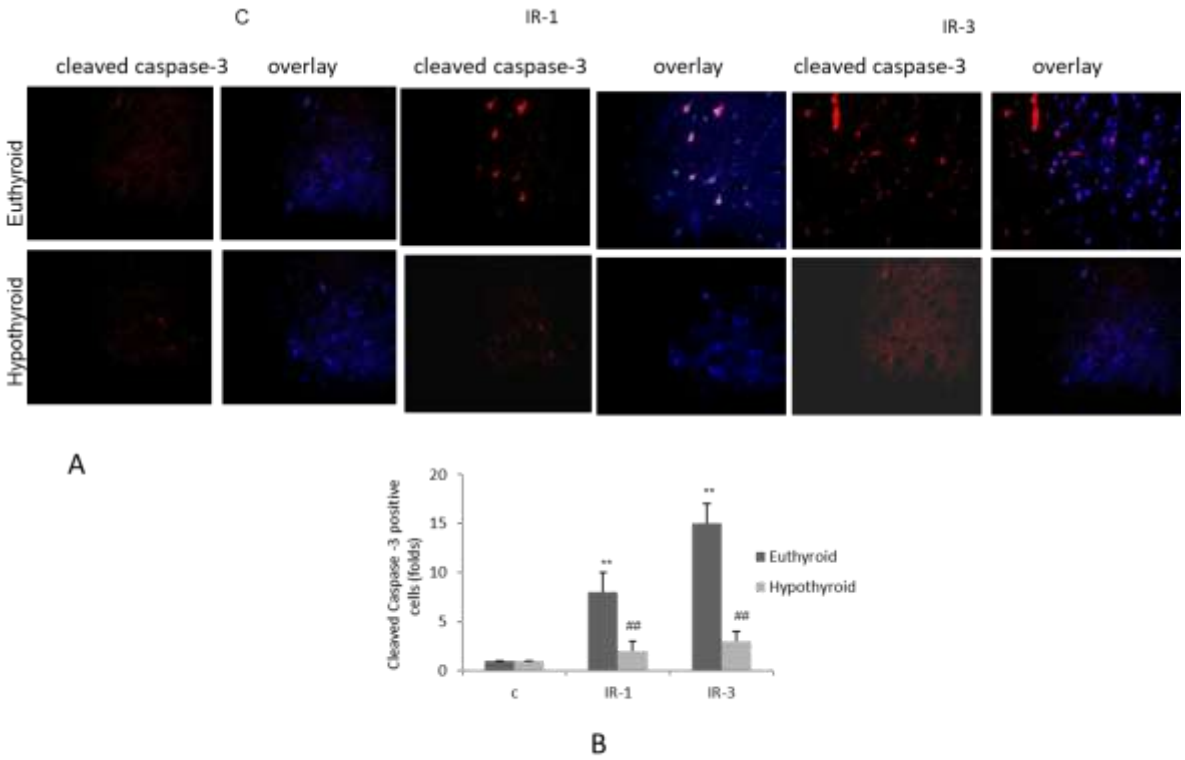

Figure 5: Paraffin embedded brain tissue sections were prepared from euthyroid, hypothyroid control C as well as ischemia reperfusion for 1 day (IR-1) & 3 days (IR-3) in both group of rats. A, Immunofluorescence for cleaved Caspase-3 (apoptotic, red) in MCA territory of ischemic cortex. Brain sections were stained with Hoechst dye and cleaved caspase-3. In the merged figures (overlay panel), pink colour indicates localization of cleaved Caspase-3 in the nuclei. B, Graph represent the fold changes in cleaved caspase -3 positive cells in nucleus. p**<0.01 versus control; ##p < 0.01 versus I/R