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PROTECTING ROLE OF AUNP CONJUGATED INSULIN ON PIAMATER AND GRANULAR REGION OF MICE BRAIN DAMAGED BY PTZ INDUCED SEIZURE

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ABSTRACT

Proteins and peptides play an important role in seizure prevention and neuroprotection and development of CNS. Acute increase in peripheral insulin level leads to higher CSF insulin, while chronic peripheral hyper-insulinemia down regulates insulin receptors in BBB, impairing insulin transport in the brain. Thus, increased insulin level in the circulatory pathway can affect the brain's normal activity. Nanoparticles are small enough to cross this BBB enhancing human cells' uptake but won't induce cell death. In this study, protecting ability of insulin and AuNP conjugated insulin was tested in PTZ induced seizure associated brain damages in mice. After specific duration mice were sacrificed by cervical dislocation. Brains were collected at once and were fixed with carnoy's fixatives. Brains' sections were made in rotary microtome at 5 µm thickness. Histological slides were observed under microscope. Piamater and granular region was more or less intact in insulin treated mice group and total prevention was observed in AuNP conjugated insulin administered animal model.

KEYWORDS: Excitotoxicity, Au-Nanoparticles, PTZ, Seizure, Neuroprotection, Piamater and Granular region.

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INTRODUCTION

Neurons are excitable cells and sensitive to different excitotoxic agents like PTZ (pentamethyltetrazole). Excitotoxic agents cause burst firing and seizure in animal model and depolarization in cultured neuron[1,2,3]. Excitotoxicity is a process, in which changes in calcium permeability causing Ca^{2+} influx into the neuron, resulting imbalance in intracellular Ca^{2+}, level causes the activation of lethal signalling pathways ending in neuronal death[4]. Early effects of Ca^{2+} influx include the activation of Ca^{2+} dependent enzymes and generation of reactive oxygen species (ROS)[5] which may harm the brain causing neural loss in the brain of mammal[6]. It causes prevention of myelination in early life and enhances demyelination in adult brain. Neuronal loss in brain occurs due to necrosis or initiation of apoptosis.Scientists are searching newer and newer chemicals to protect the neurons from seizure associated neural damage although different drugs are in use. Endogenous and exogenous protein and peptides plays an important role in seizure prevention and neuro-protection and development of central nervous system (CNS). Changes in the level of different neuropeptides and hormones like brain insulin, brain derived neurotrophic factor, neuropeptide Y etc cause neuronal excitability and neurodegenerative disorders. Controlled administration of these chemicals have neuroprotective roles. Stimulation of neuropeptide Y receptors suppresses epileptiform bursting or seizure which was studied by electrophysiological and behavioral studies[7,8,9,10,11]. Neuro-protective role of insulin against different neurotoxic agents was also reported[12].There is a close relationship between peripheral insulin and brain insulin level. Acute increase in the peripheral insulin level leads to higher cerebrospinal fluid insulin, while chronic peripheral hyper-insulinemia down regulates insulin receptors in blood brain barrier (BBB), impairing insulin transport in brain[13]. Peripheral insulin can access central nervous system directly through the area postrema. An increase in circulating insulin level has been shown to rapidly affect brain function. Insulin can be synthesized in cultured rat brain and released when cells were in depolarized state. Brain insulin synthesis occurs in pyramidal neurons particularly hippocampal neurons, prefrontal cortex, enthorhinal cortex and olfactory bulb but not in glia cells. Moreover, insulin like growth factor I (IGF-I) also present in the brain. IGF-I is an important modulator of brain function and promote neuronal survival during development. In adult brain, it plays an important role in neuro-protection. Brain insulin subunit differs from peripheral ones by slightly lower molecular weight and by the absence of down regulation after exposure to high insulin level. In mammalian brain two different types of insulin receptors are found: a peripheral - like type and a neuron specific type. Nanoparticles are extensively used in biomedical sciences for therapy, diagnosis etc. These are small enough for human cells to uptake but won't induce cell death. They can also cross the blood-brain barrier (BBB) at low concentrations. Translocation of different types of NPs into the central nervous system (CNS) via the olfactory pathway was reported[14,15]. Neuro-protective role of different nanoparticles was already reported by different workers. Gold nanoparticles are already studied to protect the brain from amyloid beta induced brain damage. Gold nanoparticles can bind with different protein due to its surface negativity. In the present experiment, insulin and AuNP conjugated insulin were applied in mice model to protect the mice brain from PTZ induced seizure associated brain damages.

MATERIALS AND METHODS

Citrate stabilized Gold nanoparticles Preparation

Citrate capped gold nanoparticles were prepared using hydrogen tetrachloride and sodium citrate as reducing and stabilizing agent 20 ml of 1.0 mM gold chloride was boiled in a hot plate magnetic stir and immediately added 2 ml of 1% solution of sodium citrate. The change in colour of gold chloride indicates the

formation of gold nanoparticles. Solution of gold nanoparticles was filtered with 0.22 mm syringe filter and stored in 4^0 C. Absorbance scan of gold nanoparticles are measured using UV visible spectrophotometer and peak absorption was found at 537 nm.

Conjugation of citrate capped gold nanoparticles with insulin

Pure insulin of concentration 40 IU/ml was diluted with distilled water in the ratio of 1:9. Diluted insulin 10 µl was mixed with 100 µl of already prepared gold nanoparticles and incubated at 4^0 C for 24 hours. Mixture was centrifuged (Eppendorf Refrigerated Centrifuge; Model 5424R) at 5000 rpm at 4^0 C for 30 minutes, supernatant was discarded and precipitate was dissolved in distilled water and analyzed the gold nanoparticles conjugated insulin using UV visible spectrophotometer (Eppendorf, Biospectrometer Basic).

Animal Experiment

Experiment was conducted in albino mice. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Gauhati University. Albino mice of both sexes of body weights between 25-30 grams were used for the experiments. The animals were handled daily to reduce stress due to manipulation. Animals were divided into four different groups, each containing six animals. The first group was considered as a control group. In the control group normal physiological saline was administered daily through subcutaneous route. The other groups were test groups and animals were treated with PTZ as a convulsant at the dose of 65 mg/Kg BW through subcutaneous route. This dose of PTZ was sufficient to induce tonic clonic seizure. Mice were carefully monitored after administration of test compounds. The PTZ treatment was continued for seven days to seizure induced brain damage. The first test group was PTZ group. Second test group was insulin treated group and insulin was administered at the dose of 8 IU/Kg BW. The third test group was insulin conjugated with gold nanoparticles treated group and treated with goldnanoparticles conjugated insulin at the dose of 1.6 IU/Kg BW.

C reactive protein (CRP) level study

For studying the serum CRP level, mice serum was collected from all the groups and stored at 2^0 - 8^0 C till the value estimated. CRP value was estimated using the “FULL RANGE CRP (frCRP) Immunoturbidometric Assay” RX DAYTONA PLUS RX MONACO kit.

Histological study of mice brain

For histological study, mice were killed by cervical dislocation and brain was collected within five minutes. Brain was fixed in carnoy’s fixative at 2^0 - 8^0 C for 24 hours. Sections were made at 5 micron thickness. Sections were stained in eosin and haemotoxyln stain, mounted in DPX and observed and microphotographs were taken with the help of microscopic camera.

RESULTS

Spectroscopic analysis

UV spectra of gold nanoparticles shows maximum absorbance at 537 nm. Insulin peak absorbance is 282 nm. After conjugation with gold nanoparticles insulin peak absorbance changes towards red. Shift of absorbance towards red clearly indicates conjugation of gold nanoparticles with insulin.

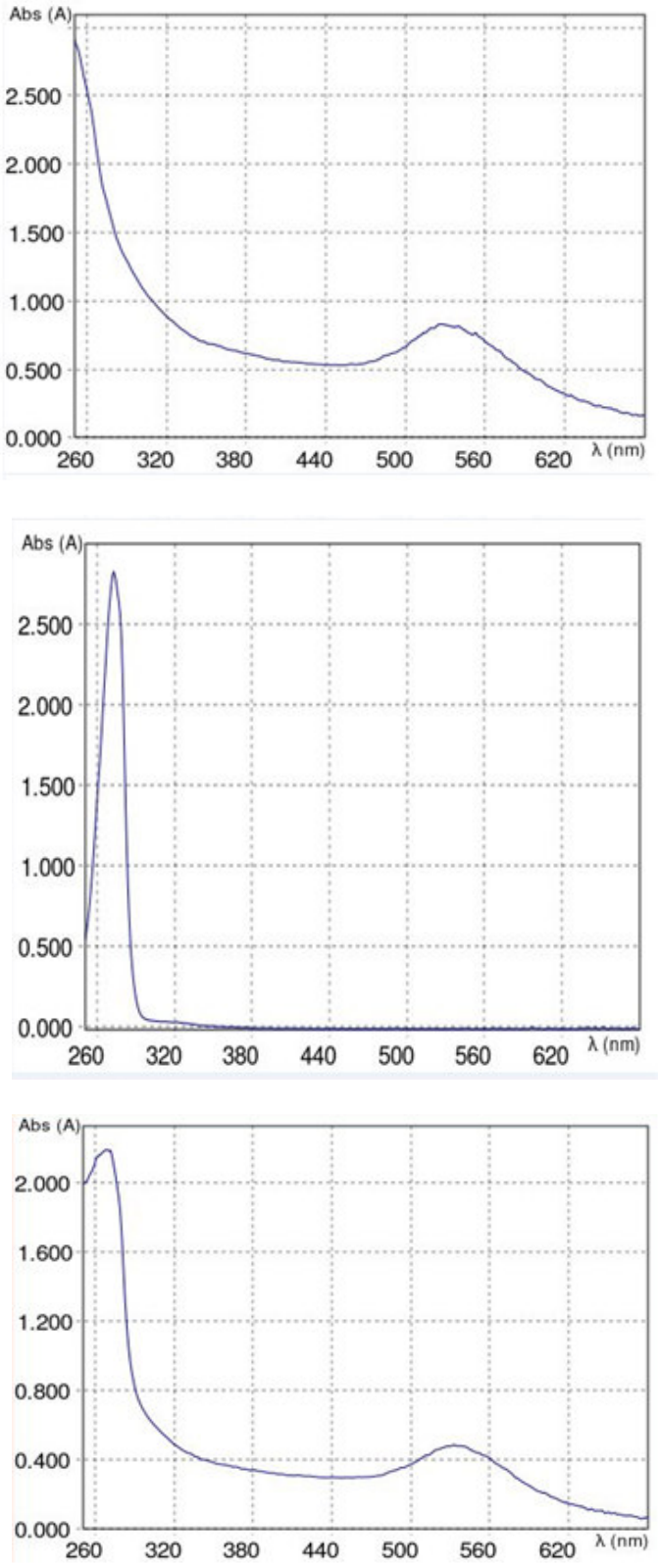

Figure 1
Graphs showing the AuNP,insulin and Aunp conjugates respectively

First graph shows the peak of the AuNP at 537 nm at a distinct concentration of AuNP; second graph shows the insulin peak; but in the third graph both the peaks of insulin and AuNP are diminising.

CRP as the Inflammatory Marker

Serum CRP level elevated significantly in PTZ treated mice. No significantly lower level of CRP was observed in insulin treated mice while significantly lower level was observed in AuNP conjugated insulin treated mice.

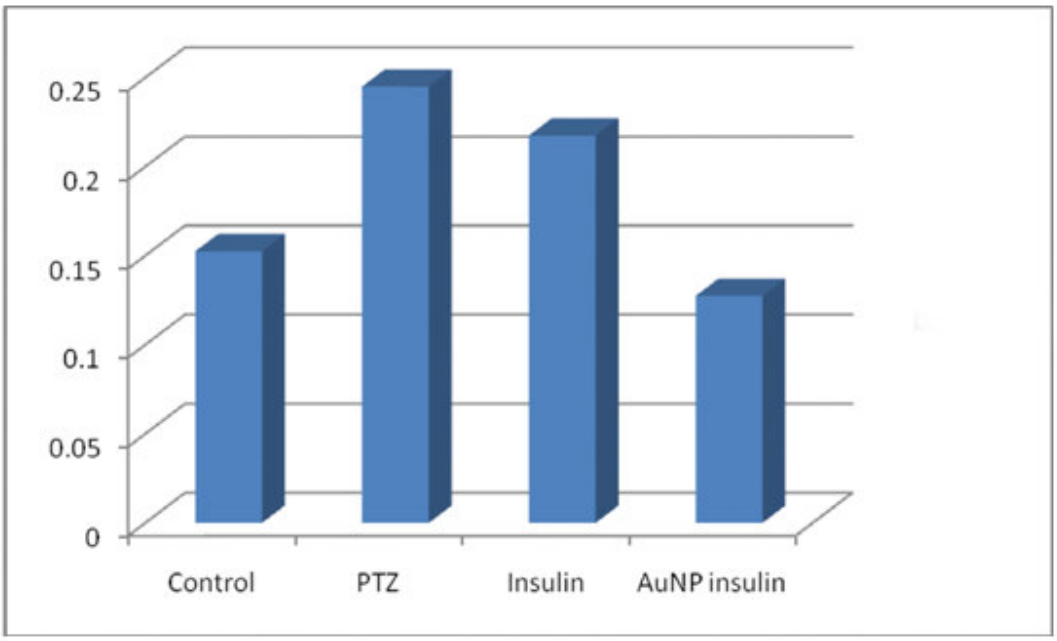

Figure 2
Serum CRP level

Serum CRP level of PTZ induced mice group is significantly increased; but after insulin administeration the level is not significantly reduced; but in AuNP conjugated insulin administered group significantly lowered level of serum CRP level is observed.

Histological findings

Histological observation clearly reveals the remarkable loss of cellular architecture in pia matter and granular layer of neocortex I region in the PTZ treated mice brain. Losses of eosinophilic materials with necrotic cells are also observed. Minimum loss of eosinophilic material with very less number of necrotic cells and intact cellular integrity in piamater region in insulin treated and also goldnanoparticle (AuNP) conjugated insulin treated mice brain. But AuNP conjugated insulin showed more efficiency in protecting the histological architecture of the brain. Loss of neurite, pattern of cellular distribution also changed in PTZ treated mice in neocortex region. Presence of pyramidal cells also observed in neurocortex I region. AuNP conjugated insulin administration protects the layer of pia matter and thus protects the superficial layer of neocortex in PTZ treated mice brain.

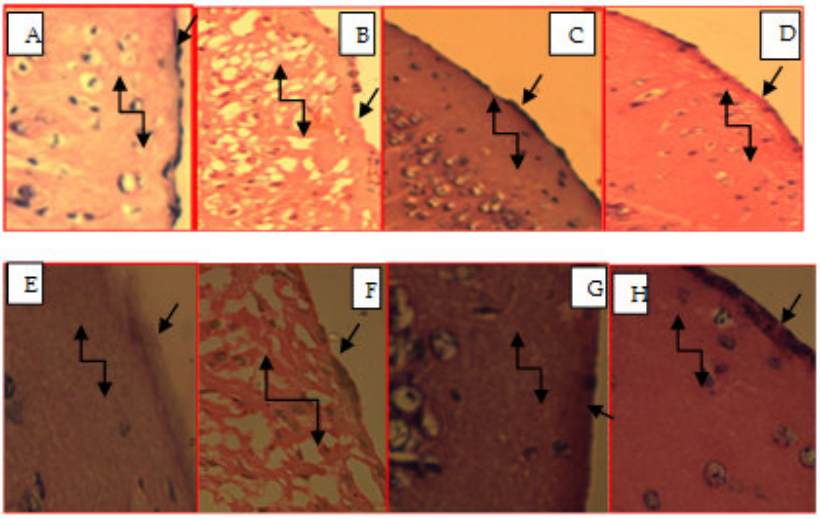

Figure 3

Grannular and pia matter region of mice brain;(A-D:10X magnification;E-H:50x magnification); A&E. control mice, B & F. PTZ induced seizure mice, C & G.Insulin administered followed by PTZ administration, D & H Gold nanoconjugated insulin administered followed by PTZ administration

" ↗ " indicates the granular region of the brain and " ⌐↑ " indicates piamater region.

DISCUSSION

Pia matter is a structure immediately covering the brain thus regulating entry of different chemicals to the brain. Loss of cellular integrity in pia mater is remarkably important for underlying brain structure and function as it enhances different harmful chemicals to come in contact with brain. Direct exposure of neuron, neurosupporting cells with different chemicals might cause the loss of cells in granular layer. Neurons and neuronal circuits undergo extensive structural and functional remodeling in response to seizure. Axonal sprouting is a prominent feature on brain development and is essential process in the establishment of neuronal connections and formation of neural circuits. Formation of neural circuits and their organization into complex networks involves a coordinated sequence of overlapping cellular events that include cell birth, differentiation, migration, neurite outgrowth or sprouting, synapse formation and programmed cell death and activity dependent pruning that refine neural connections. PTZ destabilizes the neuronal membrane and inhibits GABA level. It also causes increase in chloride ion conductance through opening of the chloride ion channel. Calcium ions play an important role in neuronal deformation. Entry of calcium ions causes release of enzymes and free radicals that destroy neurolemma. PTZ causes the release of excitatory neurotransmitter upto toxic level that affects the sodium channel. Entry of excess sodium ions may cause the swallowing of nerve terminal and finally disruption of axon terminal. Neuroprotective role of insulin was studied by different workers[12]. Insulin stimulates Akt inhibition apoptosis. Insulin protects the neurons by inhibiting neuronal and non-neuronal apoptosis via MAPK signaling and suppression of caspase3 activity. This was further confirmed by other research work P13K/Akt and ERK ½ signaling pathways[16].Presence of vacuolar region is a characteristic of autophagic cell

death in the brain region. In the present experiment large number of vacuoles in neocrtex I region demonstrated the autophagic cell death after PTZ administration. Mice pretreated with insulin and gold nano particle conjugated insulin clearly demonstrated neuroprotective nature of both the chemicals. Previously it was reported that seizure induced neural degeneration was a necrosis process as no significant change in apoptotic marker detected after administration of kainic acid to induce seizure. Possibility of new cell death mechanism due to excitotoxicity of neuron is also proposed[17]. Cell swelling, cell membrane rupture, integrity lost, partial chromatin digestion, destruction cell organelles in different neuron in neocortex I region clearly indicated necrotic cell death after excitation with PTZ in PTZ treated mice. Lower concentration of insulin is required to protect the brain after PTZ insult when insulin is conjugated with gold nanoparticles. This finding might be associated with enhance permeability of insulin through blood brain barrier or increase signaling after conjugation with negatively charged gold nanoparticles.

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