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**Research Article**

# Activity of a novel Selena-diazole derivative compound on primary neuron cells isolation from cortex and hippocampus of 18th day aged fetus of pregnant Wister rat

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## ABSTRACT
Seleno-cysteine amino acid is originated in the active site of all selenium containing enzymes such as glutathione peroxidase. Several selenoproteins in the brain, but their roles were remained not well documented. The aim of the study is to investigate the effect of a novel selena-diazole derivative i.e. 4', 4'- (4, 5, 6, 7- Tetrahydro- [1, 2, 3-] selenadiazolo [4, 5e] pyridine - 4, 6 - diyl) bis (benzene-1, 3-diol) (SeD) on viability of cortical neurons and primary hippocampus cells isolated from prenatal rat pulps at 18th days. Fetal brains were removed and placed in an ice chilled Hibernated E. Hippocampi and cortices were separated. Papain 0.05% added for 10 min at 37° C; then trituration by fire polished-glass pipettes were done. Culture dishes with diameter 60 mm or 24-well plates were coated by Poly D Lysine on a density $5*10^4$/dish. Neurons were cultured on neuro-basal medium complemented with B27 and incubated in a humidified $Co_2$ 5% atmosphere incubator at 37° C. SeD in different concentration were added, viability percentage were estimated at zero time, after 1hr, 2hr, 3hr. The results illustrated that a significant decreased in viability in a dose dependent and a time dependent manner in comparison with control and solvent groups. Despite the statistically significant level of cell viability, the levels remain high compared to other selenidiazole derivatives, also it showed the most reduction percentage after incubation for 3 hour of all treated groups, also, high significant reduction observed in 400 and 500 µg/ml treated groups. It can be concluded that SeD compound has a dose and time dependent reduce in cell viability; but still more preserve effects on neuron cells than control groups.

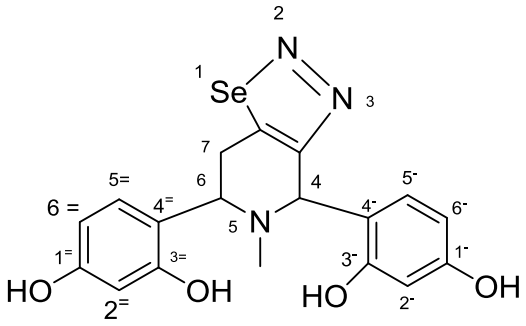
**Keywords:** Selenadiazole, primary neuron cells, viability, trypan blue, Acridine Orang Stain.

## INTRODUCTION
Detection of Selenium (Se) as a unique element in rats, which is a vital micronutrient, formed several antioxidant enzymes that have decisive roles in scavenging activity against reactive oxygen species and oxidized macromolecules, therefore selenium and selenoproteins deficiency has been associated to various complaints in humans. It was been speculating whether a minor reduction of Selenium content in a brain during long term may precipitate neurodegeneration in humans (Wirth et al., 2010). Requirement of this trace element is due to the formation of 21st amino acids, selenocysteine. Selenocysteine amino acid is originating in the active site of all selenium containing enzymes e.g. selenocysteine is critical to the glutathione peroxidases catalytic activities, thioredoxin reductases, iodothyronine deiodinases and methionine sulfoxide reductases (Fernandes & Gandin, 2015).

Several selenoproteins in the brain, but their roles in neuronal function were remained not well documented. Concentrations of Selenium in different regions of an adult brain have been described. Expression of Glutathione peroxidase in glial cells increased nearby the injured area in Parkinson's disease and occlusive cerebrovascular disease, reliable with its defending role against oxidative injury. Selenoprotein P possesses antioxidant actions and has ability to stimulate neuronal cell survival. In cell culture and gene knockout models provision a role for selenoprotein P in transport of selenium to the brain. Selenoproteins W thioredoxin reductases, type 2 iodothyronine deiodinase and 15-kDa selenoprotein, also identified in the brain(Fernandes & Gandin, 2015).

Selena diazole derivative i.e. 4', 4'- (4, 5, 6, 7-Tetrahydro- [1, 2, 3-] selenadiazolo [4, 5e] pyridine - 4, 6 - diyl) bis (benzene-1, 3-diol) (SeD)

compound was previously synthesized by the authors. FT-IR spectra, melting point and TLC were measured in the department of Pharmaceutical Chemistry, College of Pharmacy/ University of Basrah/ Iraq. 1H-NMR and 13C-NMR spectra of (SeD) compound was recorded using Bruker Ultra shield spectrophotometer (300 MHz), in University of Al-al-Bayt, Jordan. Median lethal dose and antimicrobial activity also measured. The study of synthesis and characterization, in addition to median lethal dose and antimicrobial activity was published (Neamah, Khudair, & Al-Jadaan, 2020). Also the effects of the test compound on liver, renal and lipids biochemical tests also studied (Neamah, Naaeem Khudair, & Al-Jadaan, 2019).




$C_{17}H_{15}N_3O_4Se$ (SeD)

Prime culture of hippocampal or cortical neurons of the rodents is one of the essential procedures of recent neurobiology. Primary neurons definitely collected and differentiate to a culture with separate axons dendrites, synapses, and dendritic spines. Primary culture does not divide in culture therefore it generated from either embryonic or initial postnatal brains (Sahu, Nikkilä, Lågas, Kolehmainen, & Castrén, 2019). Primary neuronal cultures can form from all parts of the brain; the most popular cultures are hippocampal cultures because of the relatively a clear architecture of the population of the nerve cells in the hippocampus. Hippocampal cultures are specially synthesized from embryonic tissue at the late-stage, which is easy to dissociate and have fewer glial cells in comparison with mature brain tissue. Hippocampal neurons Isolation from embryonic tissue reduce damage of the axons and dendrites due to less adhesion contacts. Hippocampal cultures are mostly formed from rats because hippocampus isolation is relatively easy, also a tissue isolation is done from mice with proper care (Sahu, 2008). Defective of the cell lines achieved from the precursors of nervous system due to failure of the neurons that result from these cultures to summarize central neurons features, including the formation of specific axons, synapses and dendrites. As an alternate the procedures of primary-cell cultures have been effectively accepted to in vitro study of these neurons (Beaudoin et al., 2012).

Testing the viability of the cells is a significant test in several areas of cell-study including; in tissue and cell cultures cytotoxicity tests, in regenerative medicine for selection of appropriate tissue scaffolds, in transplantation for the products quality assurance, and in cancer treatment studies. Cell viability can be evaluated microscopically, flow cytometry and estimation of cellular secretion products (Kummrow et al., 2013). The test of dye exclusion is used to determine the presence of viable cells in a cell suspension. The principle of this test is that live cells have integral cell membranes that eliminate some dyes, like Eosin and trypan blue, while dead cells keep such stains. In this test, dye mixed with the cell suspension and then examined visually to decide if cells take up or eliminate dye (Strober, 1997). Cell counting and viability estimation are usually computed, whatever the aim of a research in cell biology. The Trypan Blue assay (TBA) was obtainable about a century ago and is still the most broadly used technique to achieve cell viability analysis (Piccinini, Tesei, Arienti, & Bevilacqua, 2017).

Popular blue fluorescent dyes such as Hoechst and DAPI are nuclear-specific dyes used to stain both live and fixed cells. In solution, these dyes have insignificant fluorescence, however when they bound to DNA converted to brightly fluorescent, both dyes are nontoxic, stable and extremely permeable to cell membrane (Atale, Gupta, Yadav, & Rani, 2014). They extensively used for example nuclear counterstains and cell cycle studies for both live and fixed cells (Atale, Gupta, Yadav, & Rani, 2014). DAPI is a prevalent counterstain of the nucleus and chromosome in multicolor fluorescent procedures. Although the dye is cell impairment, higher concentrations will enter a live cell. DAPI used mainly in higher concentration than Hoechst for staining live cells due to relatively low cellular membrane permeability Tarnowski, Spinale, & Nicholson,

1991). Complex of DNA-DAPI when excited by ultraviolet light discharge strong bluish white fluorescence (Ryazanova et al., 2011); therefore very small amounts of DNA for example those in mitochondria and virus particles can be visualized. Furthermore, DAPI stain is very simple procedure and requires no hydrolysis (Hamada & Fujita, 1983).

## Materials and methods

### Materials

SeD was synthesis and characterized by the authors in pharmaceutical chemistry department/College of Pharmacy (Neamah, Khudair, & Al-Jadaan, 2020), SeD dissolved in DMSO 5: 95 Distilled water (DW).

Acridine orange, Amphotericin B, Gentamycin, Fungizone, DAPI solution, Trypan blue, 24 well plates, Round cover slips, Petri dish and double deionized distal water ($ddH_2O$) were provided from Beijing Solaria science –China. Hibernate HE, Hibernate HE-Ca, NB Active1, papain, Poly – D-lysine cover slips, Polish pasture pipette, were from Brain Bit –UK. Normal donkey serum, Poly-D-lysine, Poly-L-ornithine, Donkey anti-rabbit FITC, Fibronectin and Cell dissociation sieve were provided from Sigma-Aldrich .USA

### Animal's preparations

Ten Male and twenty female Wister rats were procured from the animal house of the Veterinary Medicine College/ Basrah University, one male and two females kept in each polypropylene cage padded with sawdust. The rats were carefully observed; presence of sperms in the vaginal swaps indicates mating and recorded as first day of pregnancy.

## Methods

### Tissue cultured

At the day $18^{th}$ of pregnancy the female rats anesthetized using chloroform, the rats abdomen carefully sterilized and the uterus and embryos were extracted. According to protocol of(Pacifici & Peruzzi, 2012), cortical and hippocampus were isolated and 37 $^0C$ and 5% $Co_2$ for 7 days Then the effect of SeD on neuron cells is examined (Sahu, 2008). Using rats as neuronal cell culture model was accepted by the Institutional Animal Care

### Pre-dilution cell count and viability test

Trypan Blue considered as the standard method for estimating the cell population mass pre dilution (Belini, Wiedemann, & Suhr, 2013). Trypan blue 0.4% dilutes with PBS to 0.2% then filtered with 0.2$\mu$ filtered. The cell suspension mixed with trypan blue 0.2% in rate 1:1, the counting of cells using counting chamber slide.

### Time and Dose dependent estimation of SeD effects on isolated Cortical and Hippocampus cells

Ten $\mu$L of SeD in concentration of 100, 200,300, 400 and 500$\mu$g/mL were added to cultured neurons, also as a control group with no treatment and solvent(v)treated group (DMSO 5%in 95% DW) were used. The time effects also measured on neuron cell culture at zero time, 1 h, 2h and 3 h. The results evaluated by alteration in cell morphology. Each concentration tested 3 times.

### Viability of neuron cells using Acridine Orang Stain

Equal volume of Acridine Orange and phosphate buffered saline PBS or Hank's balanced salt solution (HBSS) added to cultured cells. Then using fluorescence microscope the cells immediately examined and different photos were captured.

### Survival or viability percentage= (live cells/total cells)*100

Live cell stained with green, dead cells stained with red. Total cells equal to both dead and live.

### Immunostaining of neuron cells using DAPI Stain

Treated cells were fixed with 3.7% formaldehyde for 15 min and incubated with 1 $\mu$g/mL DAPI for 20 min at 37°C. Cells were then washed three times with phosphate buffered saline (PBS) and examined under a fluorescence microscope (Zhang et al., 2014).

### Statistical analyses

Experiments were carried out at least in triplicate, and results are expressed as the mean±S.D. Statistical analysis was performed using the SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, U.S.A.). Differences between two groups were analyzed using the two-tailed Student's t test, whereas those between three or more groups were analysed using one-way ANOVA comparisons. Differences with $p<0.05$(*) or $p<0.01$(**) were considered statistically significant.

## Results

### Pre-dilution cell count and viability test

Viability percentage of mature neuron cells was measured via trypan blue stain; the results revealed high viability value was (83.57), as in the table (1) and the figure (1)

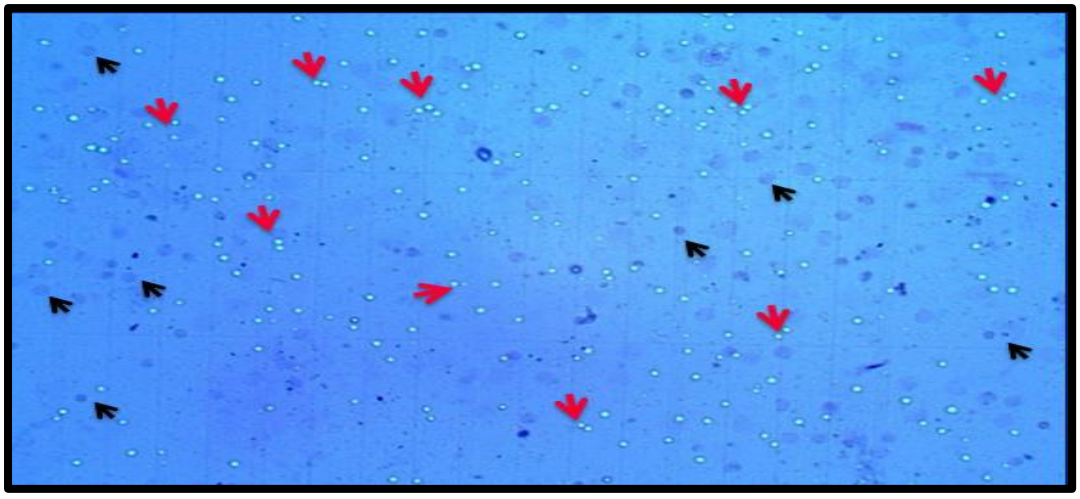


**Fig.(1): trypan blue dye exclusion test. Exhibited viable cells (red arrows) and non-viable cells (black arrow).400x**

**Table (1): illustrates the calculation of viability percentage of mature neuron cells using Trypan-Blue stain.**

| culture | Live cells | Dead cells | Total cells | Viability% |
|---|---|---|---|---|
| 1 | 775 | 210 | 985 | 78.68 |
| 2 | 341 | 67 | 408 | 83.57 |
| 3 | 373 | 104 | 477 | 78.19 |
| Average | 496.33 | 127 | 623.33 | 80.15 |
| SDEV | 241.86 | 74.22 | 315.1 | 2.97 |

**Dose- Time dependent injury of mature neurons**

Mature hippocampi and cortical neurons cultures incubated for 7days, and then treated with different concentrations of SeD 100, 200, 300, 400 and 500(μg/mL). The viability of neuron cells was measured via the inverted microscope at Zero, 1, 2 and 3h. The toxic effect of SeD using inverted microscope was investigating and compares the effect with non-treated cells as in figure 2. SeD at doses100, 200and 300 the results were essentially decreased ($p<0.05$) in number of cells without de attachment of tissue from the media and the cells not enlarged or lysis. SeD 400 and 500 groups showed de-attachment of neuron tissue and the cells enlarge in size and lysis.

According to the dose effects, solvent treated cells (V), 100, 200, 300 showed non-significant reduction in viability percentage than non-treated control group, while SeD400 and 500μg/mL treated groups were significantly reduced in viability percentage. The effects of SeD concentrations on cell viability, all treated groups significant reductions in cells viability after 2hr and 3hr, while after 0 and 1 hr the results only 500 and to less extant 400 treated group showed decreased in cell viability as illustrated in table 2 and figure 3.

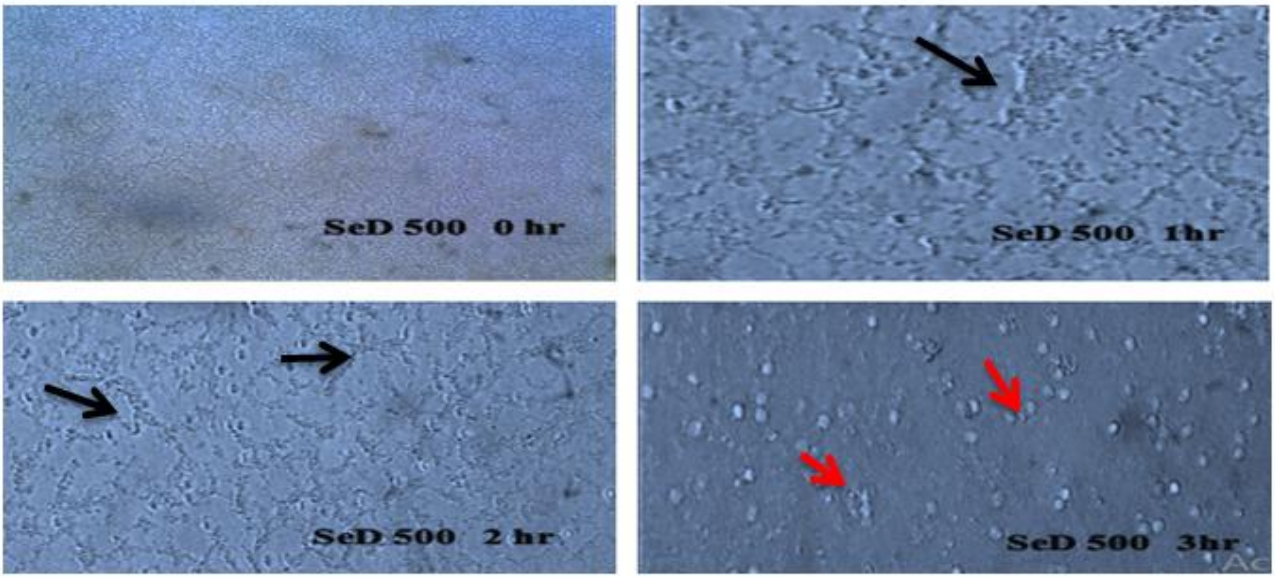



Figure 2: After one hour group treated with 500 µg/mL of SeD showed deattachment (black arrows) and enlarge in neuron cells (red arrows), Using Inverted Microscope 400x.

**Table 2: The dose and Time dependent effects of SeD on percentage of cells viability on Cortical and Hippocampi Neurons Cells**

| Time groups / Dose(µg/mL) | Zero time | 1h | 2h | 3h | LSD |
|---|---|---|---|---|---|
| Control | 92.54±4.53 Aa | 92.12±3.64 Aa | 87.9±1.55 Ab | 85.9±3.73 Ab | 4.2 |
| Solvent | 90.95±2.79 Aa | 90.04±3.08 Aa | 87.68±5.58 Aa | 84.04±5.6 Ab | 6.0 |
| SeD100 | 90.26±3.52 Aa | 88.96±5.9 Aa | 84.6±5.18 Ab | 80±5.02 Ab | 5.67 |
| SeD 200 | 90.04±3.68 Aa | 88.49±6.14 Aa | 81.8±4.75 Ab | 78.8±5.58 Ab | 6.69 |
| SeD 300 | 89.38±4.11 Aa | 86.14±2.51 Aa | 78.5±5.47 Bb | 71.06±13.7 Bb | 10.9 |
| SeD 400 | 86.47±4.9 Ba | 83.84±5.9 Aa | 76.1±9.47 Bb | 65.7±17.85 Cc | 10.34 |
| SeD 500 | 84.08±7.06 Ba | 82.77±17.23Ba | 57.73±7.95 Db | 42.68±8.79 Dc | 15.05 |
| LSD | 5.29 | 9.34 | 8.47 | 12.9 | |

*The mean difference is significant at the 0.05 level. Capital letters represent vertical relationship, while small letters represent horizontal relationship

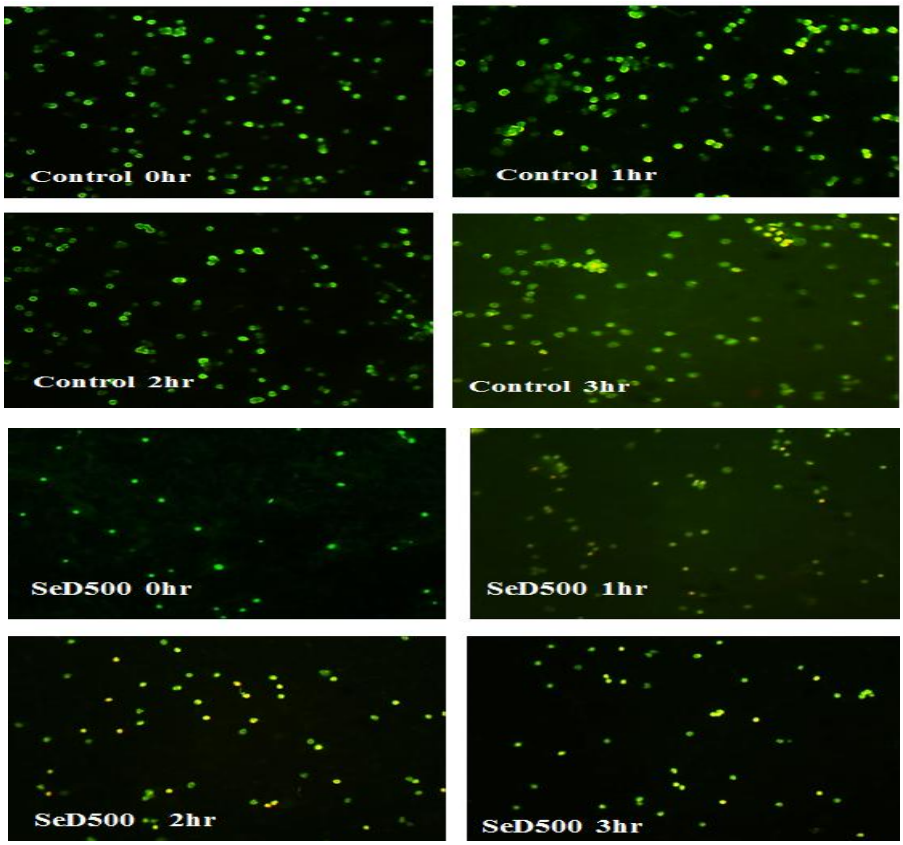



**Figure 3**: Fluorescence micrograph screening time-dependent damage of neuronal cells by acredene Dye, the cells treatment with SeD at 500µg/mL compare with control group, illustrated that Increasing the period of treatment led to decreased total number of live neurons(green) and increased total number of dead neurons (red)or (orange)after 2 and 3 h. Same experiments were repeated 3 times. 400x

### Immunostaining using DAPI Stain

Prenatal rat neurons staining as in Fluorescence micrograph (figure 4) showing the cell culture was dissociated of with green staining, beta- tubulin III which was distinguished in cell bodies and axons of these cells.

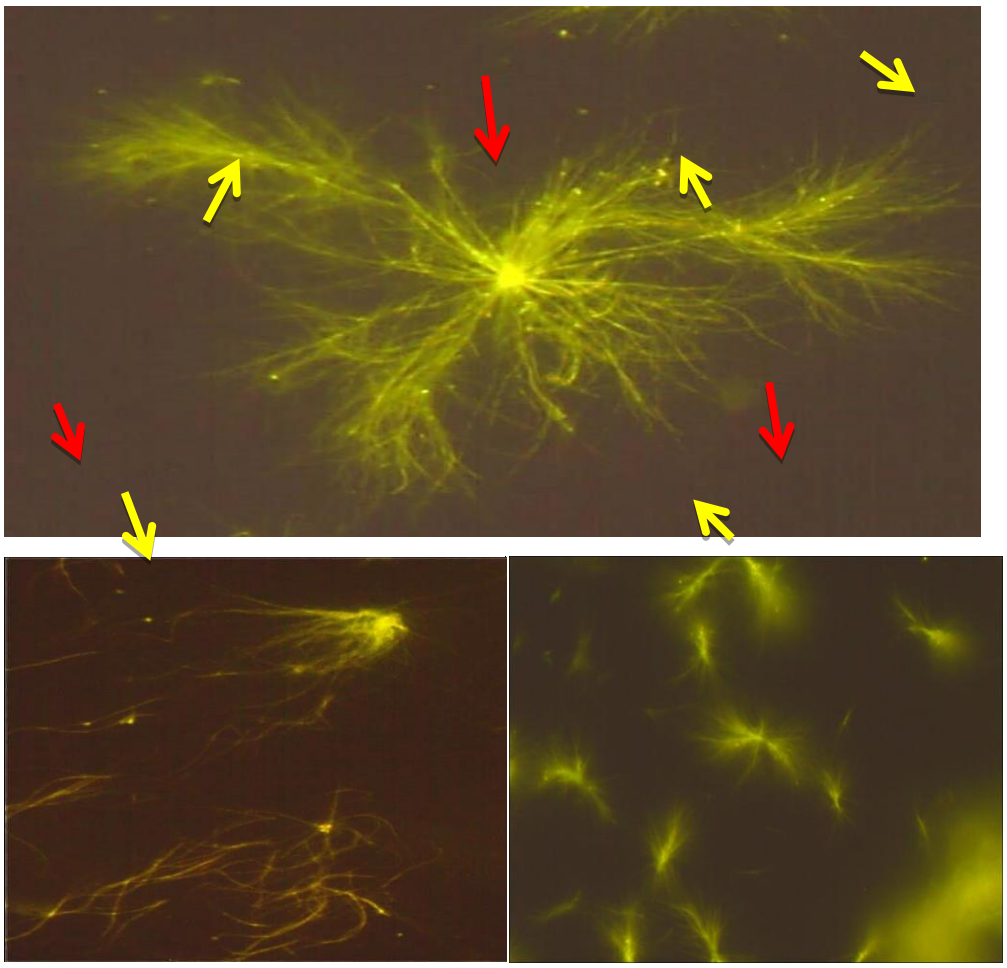


Figure 4: Immunocytochemistry/Immunofluorescence-Tuj 1 Staining Of Neuron-Specific Class III Beta Tubulin(Primary Antibody) In Differentiated Neural Cells(neurons and astrocytes). Cells Were Staining Using Conjugated Secondary Antibody Anti Rabbit FITC, Yllaow rods(Axon Of Neuron), red

## Discussion

Rodent primary culture of cortical or hippocampus neurons still one of the modern fundamental neurobiology procedures. Primary neurons can be collected easily and differentiate over a few days into a culture with obviously divisible synapses, axons, dendritic spines and dendrites. By adjusting culture conditions and medium, several factors guiding different features of neuronal existence phenotype, and differentiation have been discovered. Se has an important role in cell proliferation and apoptosis but the Se mechanisms of action are not well known. Cell cycle preserve mechanism by which the cells reproduce themselves and apoptosis cells a suicide mechanism; in both human and animals must be regulated to preserve homeostasis of the tissues (Zeng, 2009).

The importance of Se in neurological diseases pathogenesis has been revealed by a number of studies in animal, human models and cell culture that totally associate to deficiency of Se and a disturbance in brain metabolism. Future investigation is requisite to fully reveal the role of selenoproteins in neuron cells, and this will offer potential uses of selenoproteins in CNS for example biomarkers of normal or pathogenic biological procedures. The significance of Se has in preserving normal function of the brain, Se supplementation studies are developing, but publication is still indistinct concerning the precise properties different doses and of Se formulae (Cardoso, Roberts, Bush, & Hare, 2015).

Dimethyl sulfoxide (DMSO) was used as a vehicle in the present study, since the DMSO is the most commonly utilized as a solvent (Balakin, Savchuk, & Tetko, 2006). Respectable investigational design instructions that the group treated with certain drug must be compared to a vehicle treated group only. But, DMSO has different antagonistic effects, like anti-oxidant neuroprotective, anti-inflammatory (Capriotti &

Capriotti, 2012). DMSO is commonly used as a solvent but in low concentrations to avoid DMSO-mediated neurotoxicity (Julien et al., 2012). In summary, DMSO doses less than 1% are frequently neurotoxic in vitro to astrocytes and neurons. In contrast, doses ≤0.25 have slight effect on neural cell survival, or morphology within 48 h Such as, the antioxidant properties of DMSO may suppress the toxicity of a test compound.

The ability to culture and grow primary neuronal cells possesses an obligatory part of neuroscience. One of the disadvantages of primary culture is that they do not divide in culture and need to be generated from embryonic or early postnatal brains every time. Moreover, successful dissection and preparation of cultures requires substantial skill and experience. Over several decades, cell lines have been discovered and created that mimic many or most of the features of primary neurons, and more recently, differentiated stem cells from rodents or humans have been introduced as alternatives for primary cultures, but none of these have replaced embryonic primary neurons from their position as a gold standard for neuronal cultures (Saito, Yoshida, Akazawa, Takahashi, & Niki, 2003). Primary cultures permit the investigator to study precise chemical modification and treatment, cellular pathways, patterns of localization and growth in a well-controlled environment. Several of these trials utilize advanced procedure to visualize specific variations in cell responses. Hippocampal neuron cells are used to examine particular neuronal pathways that would confirm difficult to analyze in the unharmed brain. It's important to prepared homogeneous populations of neuron cells from definite brain areas for studying brain function, molecular effects can be essential in delineating pathways higher order like memory or learning. This protocol has provided comparatively pure cultures of hippocampal neurons, in absences of a feeder layer of glial cells, these neurons are used for studies of immunofluorescence. But, as with all primary culture, contamination by less preferred cells can occur (Sahu, 2008). It is familiar that selenium is vital for cell culture. Serum free media, chiefly for neurons and immune cells, contain sodium selenite, insulin and transferrin. Cells deprived of selenium cannot proliferate and survive; but the mechanism for Se role in proliferation is not well known (Tian, Su, Wu, & Zhang, 2005).

Trypan Blue dye (TBA) exclusion assay, the main manner predictable in the literature, is reflected the usual method for measurement of cell viability and the most frequently used (Chan, Kuksin, Laverty, Saldi, & Qiu, 2015). TBA is an impermeable particle to intact cell membrane and can only permit in cells having damaged membrane. Also, determination of the cell viability is by identifying of the integrity of cell membrane. Consequently when TBA passes into the cell, TBA attaches to intracellular proteins. The living cells residues color not affected and the dead cells appear blue are not prominent. TBA has a toxic effect on cells in a Time depending manner, therefore limiting cell counting after staining to only a brief period (Tsaousis et al., 2013). Binding of TBA to proteins and non-specific artifacts especially in primary cells, result irreversible cell membrane injury as result false dead cells appeared. Using a manual counting by Hemocytometer and light microscope, which consumes time, therefore fluorescence microscope and several fluorescent dyes are more consistent indicators of cellular viability. The traditional colored dyes; Propidium Iodide and Acridine Orange staining's shown to be more precise in identifying live and dead cells. Acridine Orange is a cationic dye, membrane-permeable stain that binds to nucleic acids of viable cells, under fluorescence microscopy; viable cells appear as fluoresce green, while nonviable cells appear as fluoresce orange. Although trypan blue stain is the most commonly used investigation for cell viability as it is easy to use, low-cost, it reacts rapidly and can be envision with a typical bright field microscope offered in all laboratories (Piccinini, Tesei, Arienti, & Bevilacqua, 2017).

Number of techniques for nuclear staining such as propidium iodide, DAPI, Acridine orange - Ethidium bromide staining; among others have been developed to assess the changes in DNA. Some techniques for non-nuclear staining for example Annexin-V, which does not stain DNA, however aids to detect DNA alteration and initiation of apoptosis (Atale, Gupta, Yadav, & Rani, 2014). DAPI is a general nuclear dye for cell cycle identification, definitely stains only nuclei, DAPI fluorescence dye can transmit cell membrane then; it binds toughly DNA strands, the florescence that produced 20 fold stronger than DAPI alone. DAPI cross through integral membrane therefore it can stain fixe as well as live cells (Miller, Balaram, Young, & Kaas, 2014). The fluorescence increase is supposed to by water molecules displacement from the minor groove and DAPI.

Failure of the neurons isolation and cultured from the brain of adult mammalian and the failure of the regeneration of the neurons have contributed to the conception that adult neurons do not regenerate. In the present study the effect of SeD

at different concentrations were studied on cortical and hippocampus of the rats by cell damage assay and neural culture procedure. SeD has no destructive effect on the neural cell at first three doses i.e. there were no provoked swelling and neurons injury. Selenium deficiency may initiated Cell death by increased ROS production especially lipid hydro peroxides which involved in death of the cells (Tian, Su, Wu, & Zhang, 2005).

Selenoprotein expression is controlled by Se availability such as in response to addition of Se in diet. In Se deficiency state, some proteins are synthesized at a higher scale more than other proteins. Inadequate Se results is in opposition between selenoproteins transcriptions; iodothyronine deiodinase the least influenced by low dietary Se. Selenoproteins P position on the ladder of selenoproteins transcription is intermediate (Balakin, Savchuk, & Tetko, 2006). Low selenium concentration in elderly patients related with rapid decline in cognitive functions and poor performance; but also subclinical Se deficiencies. However, subclinical deficiencies may influence cellular biotransformation and can induced several diseases including neurodegenerative disorders especially if it's related with other factors such as environmental and genetic. Besides, Se in additional is associated with pathological states. Then, the significance of preserving Se intake for adults either through food or supplements (Ellwanger, Franke, Bordin, Pra, & Henriques, 2016). The recommended daily consumption is 55μg/d for both men and women. Doses of 100-200 μg Se/d in human subjects inhibit genetic impairment and development of cancer, and the upper safe limit is about 400 μg Se/d. Selenium has a number of insulin like roles like increase cellular glucose uptake, regulation of; gluconeogenesis, fatty acid synthesis glycolysis and pentose-phosphate pathway. However High Se levels result associated with insulin resistance in mammals due to enhanced in GPx1 expression, which may affect insulin function by over production of reactive oxygen species intracellularly which are obligatory for insulin sensitizing (Zeng, 2009).

The cells viability of many SeDs were estimated using MTT assay, many studies reported that selenadiazole derivatives have anticancer activity by increase SeD uptake and induced tumor cells death. Some of the previous studies recognized in precise areas in the brain such as Cerebellum, olfactory bulb, hippocampus, olfactory bulb, cortex and choroid plexus as Se restricted regions that required to certain selenoproteins for appropriate brain function. Cortex and hippocampus neurons definite high levels of selenoproteins W1 and associated with appropriate selenoproteins P1 (Raman et al., 2013). But to the best of our knowledge there were no previous studies similar to our investigation at which normal neural cells are used to assessment the viability percentage. It can be concluded that SeD compound has a dose and time dependent reduce in cell viability; but still more preserve effects on neuron cells than control groups.

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Nil

Conflicts of Interest
There are no conflicts of interest.

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