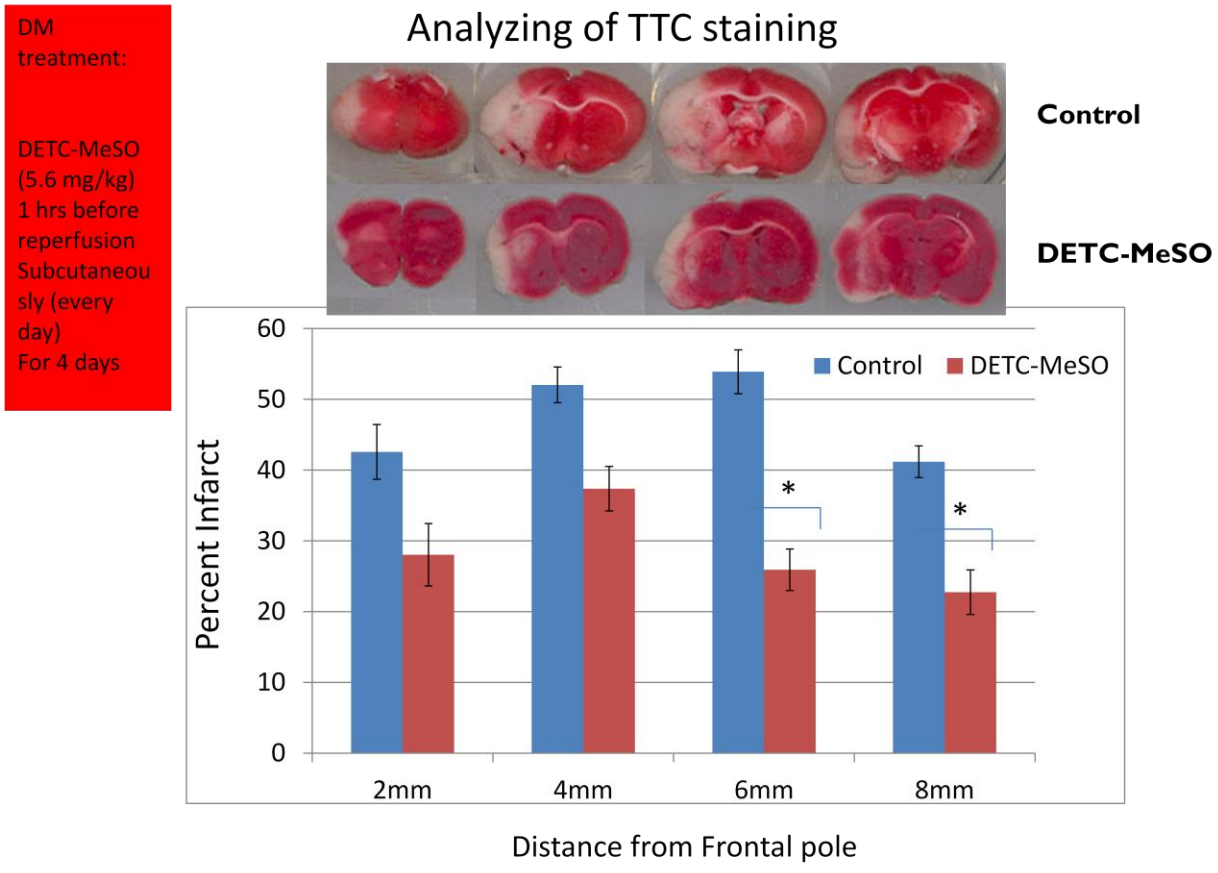
Ischemic Stroke model.

I. DETC-MeSO as a therapeutic agent for stroke treatment

DETC-MeSO at 5.6 mg/kg is effective in reducing stroke-induced brain infarction administered either before or post to ischemic condition as described below:

1). Effect of DETC-MeSO treatment prior to middle cerebral artery occlusion (MCAO) surgery and reperfusion on stroke induced brain infarction in rat MCAO stroke model

DETC-MeSO at 5.6 mg/kg was given one hour before MCAO surgery and reperfusion and then continued at the same dose for 4 days. The animals were sacrificed on 4th day after Ischemia. The brain was stained with 1 % TTC. DETC-MeSO significantly reduced brain infarct size at the dose specified (Fig1)



Data represent infarct volume as percent of the total ipsilateral hemisphere volume and are means ± SE of 21 experiments for MCAO and MCAO plus DETC-MeSO (Pretreatment). * : Significant

Fig.1: Effect of DETC-MeSO treatment prior to MCAO surgery and reperfusion on infarct size in the MCAO stroke animal model.

Upper panel: Morphology of MCAO rat brain stained with TTC. Following ischemia and reperfusion animals were sacrificed for determination of the infarct size by TTC staining. Representative brain slices from ischemia and reperfusion animals without or with prior treatment of DETC-MeSO are shown on the upper panel (No DETC-MeSO) and lower panel (+ DETC-MeSO), respectively.

Lower panel: Quantitative analysis of infarct size with and without DETC-MeSO treatment After ischemia and reperfusion animals were sacrificed for determination of infarct size by TTC staining as described in **Upper panel** Animals treated with DETC-MeSO demonstrated a decrease in infarct size by 50% at 6mm from frontal pole compared with the area of the no-drug treated group. All data were expressed as the mean ± SEM (N=21). One-way ANOVA with post-hoc Dunnett test was used to compare means between groups. Differences of $P<0.05$ were considered statistically significant.

2). Effect of DETC-MeSO treatment prior to MCAO surgery and reperfusion on the expression of Bcl-2 and Hsp27 proteins in the MCAO stroke animal model.

Bcl-2, an anti-apoptotic protein and Hsp-27, a stress and chaperone protein were found to significantly increase in both the core and the penumbra region of the infarct brain tissue in the MCAO animal treated with DETC-MeSO (Fig. 2) suggesting that DETC-MeSO may exert its neuroprotective function by up-regulating the anti-apoptotic or pro-survival proteins.

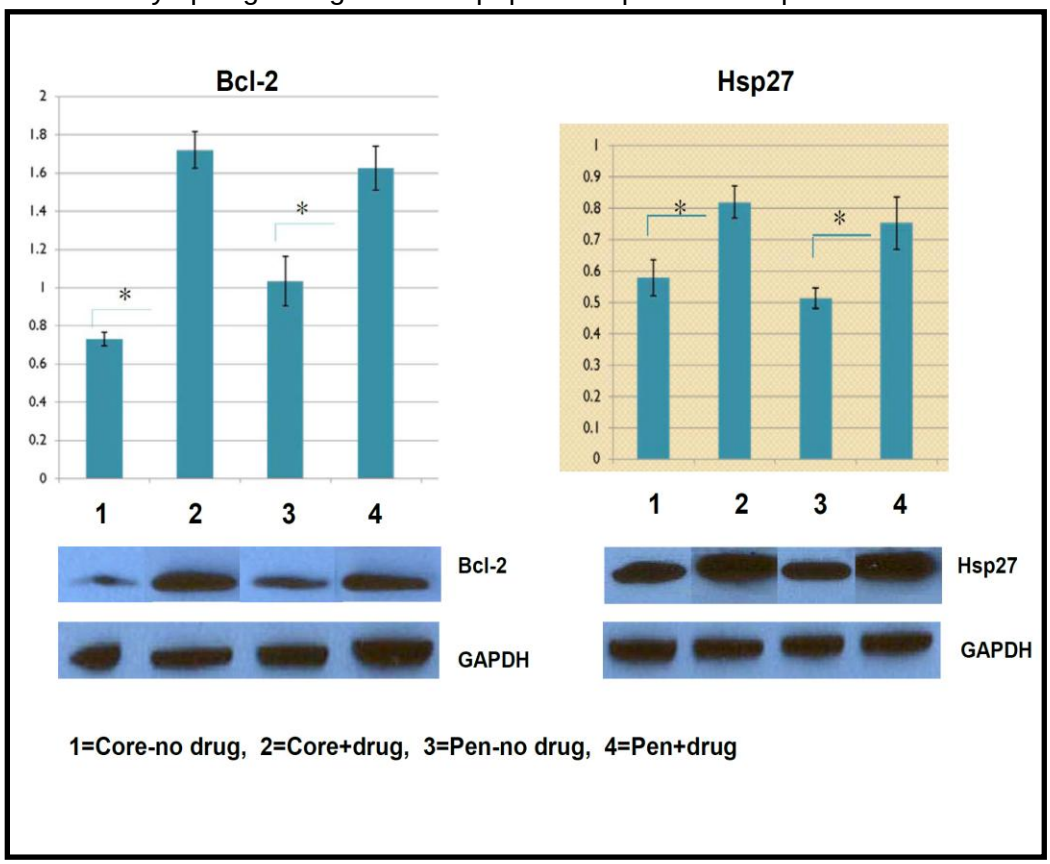


Fig. 2: Effect of DETC-MeSO treatment prior to MCAO surgery and reperfusion on the expression of Bcl-2 and Hsp27 proteins in the MCAO stroke animal model. Column 1: Infarct core; 2: Infarct core with DETC-MeSO treatment; 3: Penumbra; 4: Penumbra treatment with DETC-MeSO. A representative Western blot was presented (lower panel). Western blots were quantified by densitometric analyses expressed as arbitrary unit (upper panel). All data were expressed as the mean±SEM (N=8). Two-way ANOVA with post-hoc Bonferroni test (Prism software) was used to compare means between groups on core and penumbra with or without administration of DETC-MeSO. Differences of $P<0.05$ were considered statistically significant.

3). Effect of DETC-MeSO treatment post MCAO surgery and reperfusion on ischemia - induced brain infarction.

DETC-MeSO at 5.6 mg/kg was given by intraperitoneal injection 24 hr **post** MCAO surgery/reperfusion and by continued daily injection at the same dose for additional 4 and 8 days until the animals were sacrificed. Morphological observation based on TTC staining shows that DETC-MeSO significantly reduced brain infarct size when it was administered 24 hrs **post** MCAO/reperfusion at the dose specified in both 4 and 8 days (Fig 3, upper panel). Statistical analysis of the results obtained from 8 animals shows that DETC-MeSO treatment **post** MCAO surgery/reperfusion reduced ischemia - induced brain infarct size by 64+/-11% and 61+/-7% at 4- and 8-day respectively (Fig 3, lower panel).

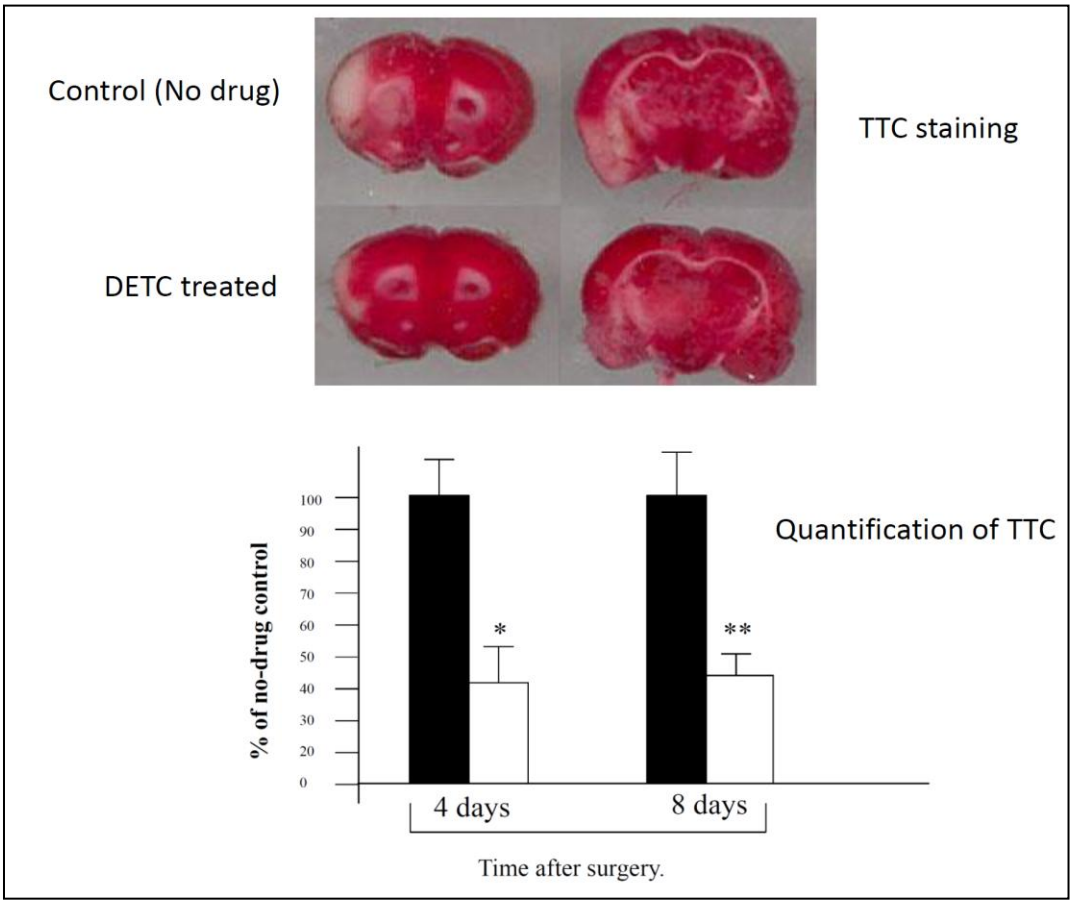


Fig. 3: Effect of DETC-MeSO treatment post MCAO surgery/reperfusion on ischemia -

induced brain infarction.
(Upper panel) - Representative TTC staining on brain slices from no DETC-MeSO treated rats and DETC-MeSO treated rats at 4 - and 8-days post MCAO surgery/reperfusion.
(Lower panel) - Size of infarction of DETC-MeSO treated group calculated as % of infarct area from no DETC-MeSO treated group. The solid black column is for the no DETC-MeSO treated group as 100% and the open column indicates the % of infarct in the DETC-MeSO treated group.

II.G-CSF as an effective therapeutic agent for treatment of stroke

We found that when G-CSF was administered 24 hours post ischemia at 50ug/kg the ischemia-induced brain infarct size is greatly reduced as shown in Fig. 4

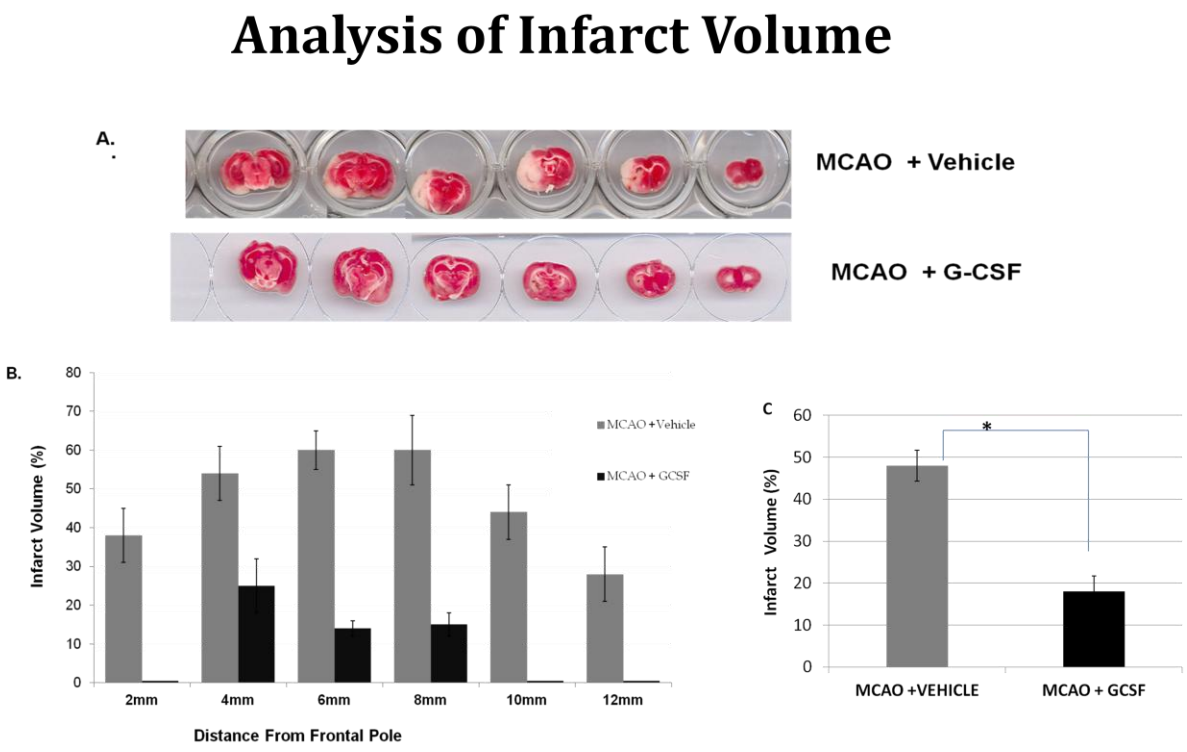


Fig 4 Effect of G-CSF on ischemia-induced brain infarction

.(A) Gross anatomy of brain sections (2mm) after TTC stain. (B) Infarct volume measured in brain slices at a distance of 2mm interval from the frontal pole. Infarct volume is significantly reduced in G-CSF treated animals (n= 5) versus control group (n=5). (C) Percentage of infarct volume in entire brain of G-CSF treated animals (n=5) versus control group (n=5). For both B and C significance of $P < 0.05$ and mean ± SE.

Furthermore, we have demonstrated that the neuroprotective function of G-CSF is partially due to its up-regulation of endoplasmic reticular (ER) pro-survival/anti-apoptotic marker proteins e.g., **pAKT; JNK;Bcl-2** or down-regulation of pro-apoptotic proteins e.g., **CHOP** as shown in Fig 5.

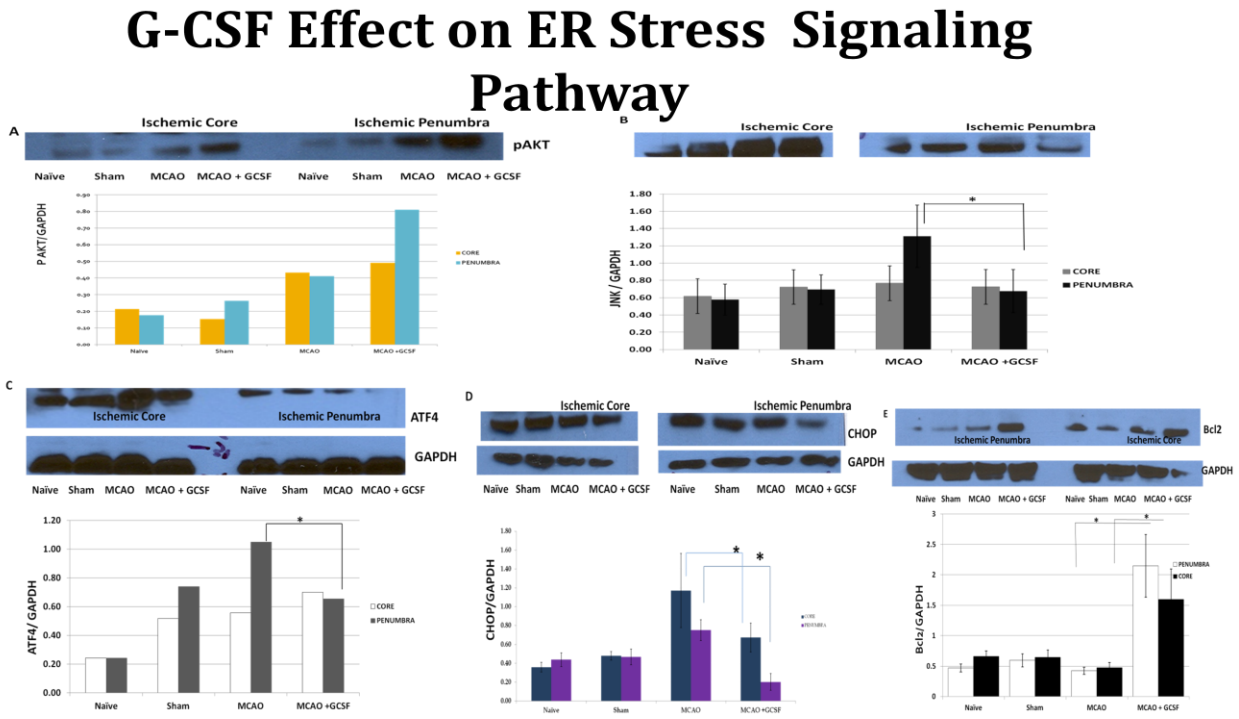


Fig 5. Effect of G-CSF on ER stress signaling pathway in MCAO rat stroke model.

A) An increase of pAKT in the G-CSF treated animals indicates that G-CSF is activating this survival pathway. The PI3-AKT pathway is one of the methods by G-CSF mediates its anti-apoptotic effect. (B) JNK (an anti-apoptotic molecule) is a downstream molecule in the IRE1α-ER stress mediated pathway. JNK is decrease in the penumbra of the GCSF treated animals. (C). ATF4, a down stream molecule in the PERK –ER stress mediated pathway is reduced in the GCSF treated animals. (C) CHOP (a-pro-apoptotic molecule) is reduce significantly in both the core and penumbra of the GSCF treated animal while Bcl2, an pro-survival molecule, is significantly increased in the G-CSF group compared to control group. Significance of $P < 0.05$ and mean ± SE.

III. Sulindac as a therapeutic agent for stroke

We found that when sulindac was given via subcutaneous injection (0.2mg/day) for 2 days before (Pre MCAO Surgery/Reperfusion) and 24 hrs after ischemia (Post MCAO surgery/Reperfusion) the infarct size induced by stroke was significantly reduced as shown in Fig.6.

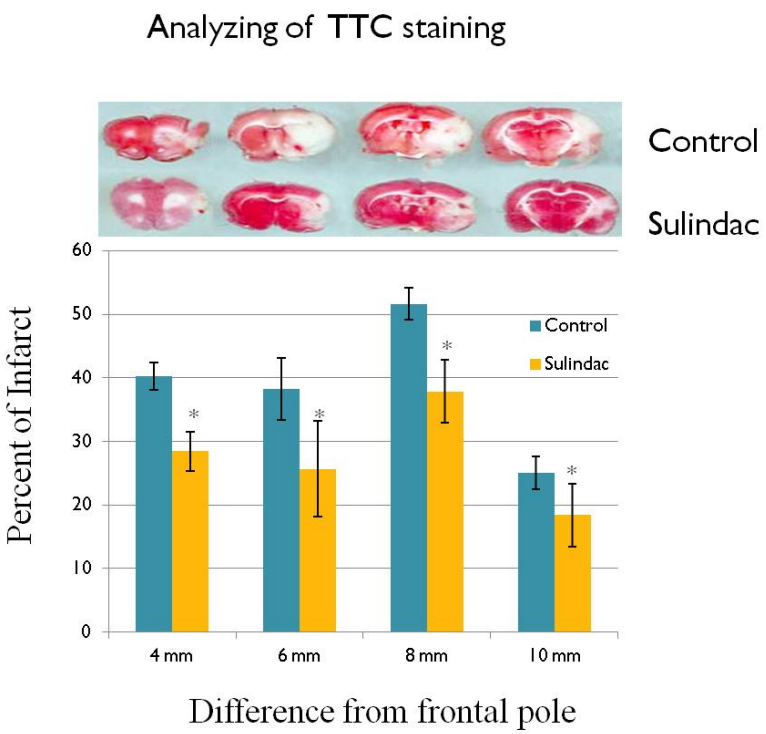


Fig 6 Effect of sulindac on ischemia-induced brain infarction

Upper panel: TTC staining of brain slices from control group and from the sulindac-treated group showing a significant decrease of infarct size in sulindac-treated group.
Lower panel: Quantitative analysis of TTC stained brain slices indicated a significant decrease in infarct size in sulindac treated animals at 4 mm, 6 mm, 8 mm and 10 mm from the anterior pole ($P < 0.01$; 2 way ANOVA).

In addition, we also found the level of anti-apoptotic protein markers such as Bcl-2 is greatly elevated in sulindac treated group (Fig. 7).

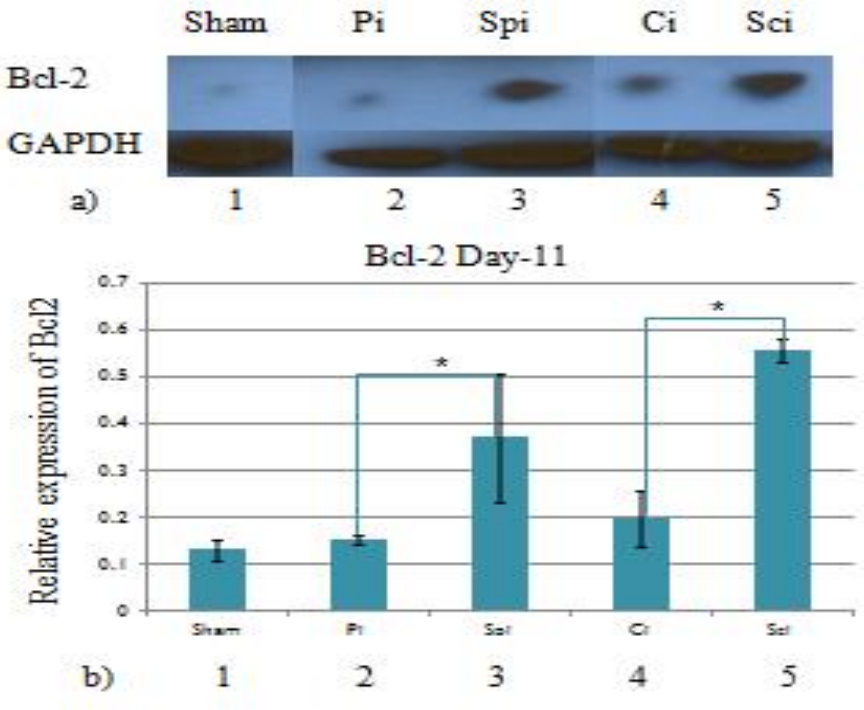


Figure 7: Effect of sulindac on expression of anti-apoptotic protein, Bcl-2, in MCAO rat stroke model.

Bcl-2 expression in penumbra and core of ischemic area of stroke on Day-11 a) Western blots staining. b) Quantitative analysis of Western blots staining. Average results (n=5) of Bcl-2 densitometric scanning are presented. 1- Sham, 2- Penumbra of control ischemic (Pi), 3-Sulindac treated penumbra (Spi), 4- Core of control ischemic (Ci), 5-Sulindac treated Core (Sci) (All data are presented as mean+/-SEM, where *p<0.05, **p<0.01, ***p<0.001)

IV. G-CSF/DETC-MeSO/Sulindac combined multi-drug treatment for stroke

We found that G-CSF/DETC-MeSO/sulindac at dose of 1/10 of the individual drug (G-CSF, 10ug/kg; DETC-MeSO, 0.56mg/kg and sulindac, 0.2mg/kg), reduced the infarct size significantly, approximately, 40%, when it was administered 24 hrs **post** MCAO surgery/reperfusion and examined at 8 days after MCAO/Reperfusion while individual drug at

such a low concentration showed no significant effect on ischemia-induced infarct size The details are described below:

<u>Experiment 1</u>- Effect of G-CSF/DETC-MeSO/Sulindac combined multi-drug treatment post MCAO surgery/reperfusion on ischemia - induced brain infarction

We found that G-CSF/DETC-MeSO/sulindac at low dose (1/10 of the original individual drug used) (G-CSF, 10 ug/kg; DETC-MeSO, 0.56mg/kg and sulindac, 0.2mg/kg), reduced the infarct size significantly, approximately, 70%, when it was administered 24 hrs **post** MCAO surgery and examined at 4 days after MCAO/Reperfusion (Fig.8).

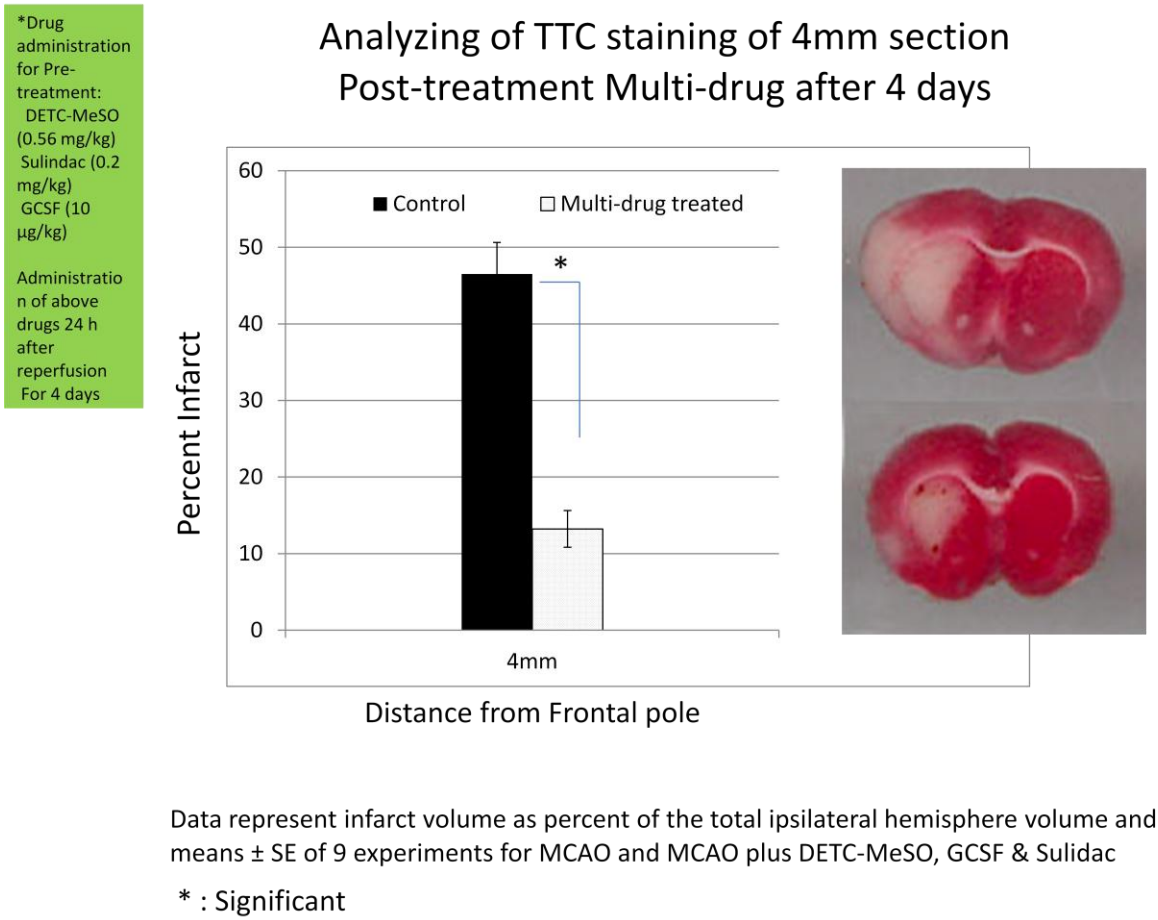


Fig.8. - Effect of G-CSF/DETC-MeSO/Sulindac combined multi-drug treatment post MCAO surgery on ischemia - induced brain infarction
G-CSF, 10 ug/kg; DETC-MeSO, 0.56mg/kg and sulindac, 0.2mg/kg which is only 1/10- of the original dose when individual drug was used as therapeutic agent for stroke were administered 24 hours **post** MCAO surgery and reperfusion for 4 days.
(Right panel) - Representative 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining on brain slices from MCAO and MCAO plus DETC-MeSO/GCSF/Sulidac combined multi-drug treated rats at 8-days post MCAO surgery.
(Left panel) - Data represent infarct volume as percent (%) of the total ipsilateral hemisphere volume and are means ± SD of 9 experiments for MCAO and MCAO plus DETC-MeSO/GCSF/Sulidac combined multi-drug treatment

<u>Experiment 2:</u> Effect of G-CSF/DETC-MeSO/sulindac multi-drug treatment post MCAO on the express of ER stress proteins and pro-apoptotic and anti-apoptotic proteins in MCAO rat stroke model.

We found that ischemia-induced pro-apoptotic proteins e.g., Bax, Bak or ER stress proteins e.g., GRP78 were reduced markedly at infarct area to an extent of 55%, 88% and 62%, respectively, in stroke animal model received G-CSF/DETC-MeSO/sulindac combined multi-drug treatment as described in Exp. 1 (Fig 9). In addition, the anti-apoptotic proteins e.g., Bcl-2 in both the infarct and penumbra regions was increased greatly to an extent of 437% and 225%, respectively. (Fig 9)

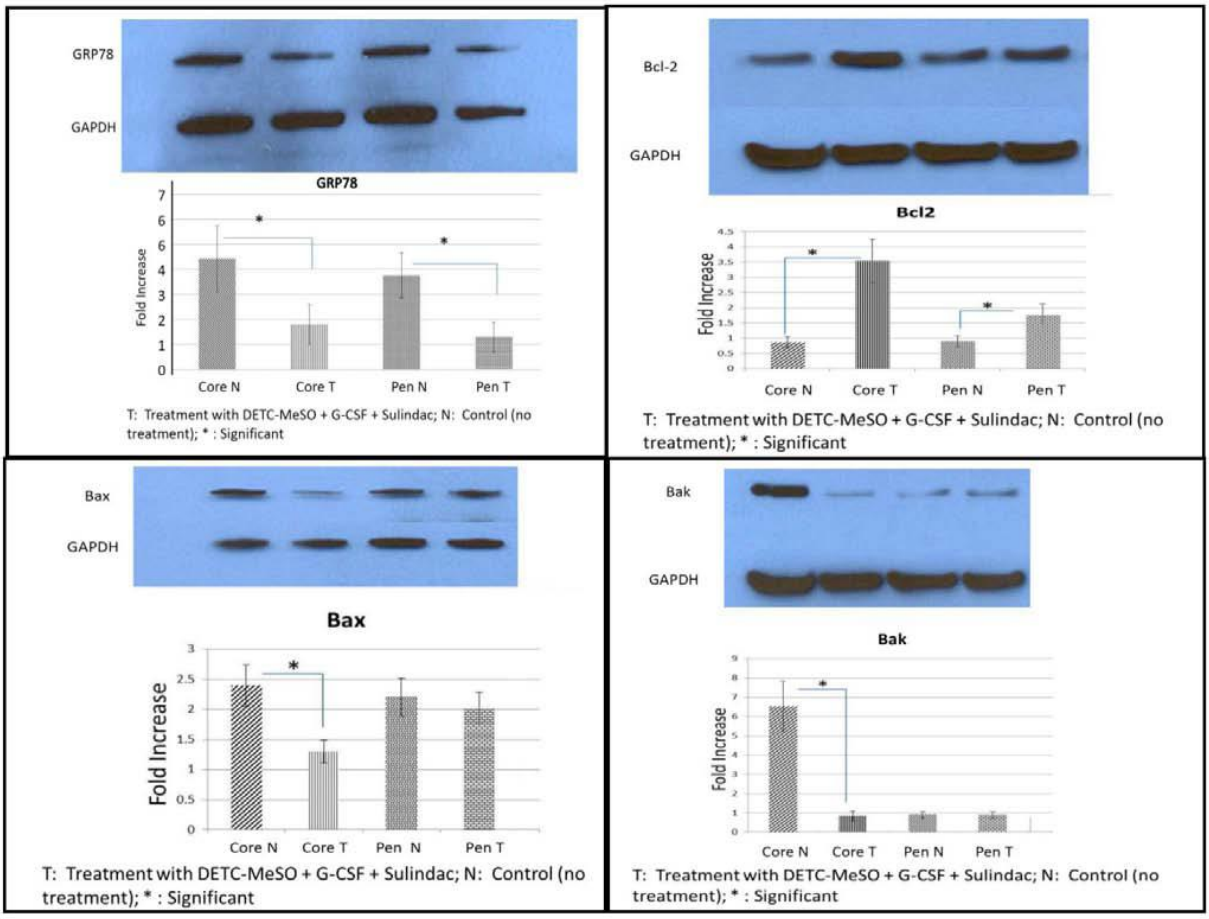


Fig.9 Effect of G-CSF/DETC-MeSO/sulindac multi-drug treatment post MCAO on the expression of ER stress proteins and pro-apoptotic and anti-apoptotic proteins in MCAO rat stroke model.

A representative Western blot of GRP78, Bcl-2, Bax and Bak is shown on the upper panel. Core N: Infarct core without drug treatment; Core T: Infarct core with G-CSF/DETC-MeSO/sulindac multi-drug treatment; Pen N: Penumbra without drug treatment; Pen T: Penumbra with G-CSF/DETC-MeSO/sulindac multi-drug treatment. Western blots were quantified by densitometric analyses expressed as fold increase using the ipsilateral un-lesioned side as the control (lower panel). All data were expressed as the mean±SEM (N=8). Two-way ANOVA with post-hoc Bonferroni test (Prism software) was used to compare means between groups on core

and penumbra with or without administration of G-CSF/DETC-MeSO/sulindac multi-drug treatment. Differences of $P<0.05$ were considered statistically significant.