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INVESTIGATION OF NOVEL FORMAZANS FOR ATTENUATION OF SUBARACHNOID HEMORRHAGE INDUCED CEREBRAL VASOSPASM IN RATS

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ABSTRACT

Cerebral vasospasm is delayed-onset cerebral arterial narrowing in response to blood clots left in the subarachnoid space after spontaneous aneurysmal subarachnoid hemorrhage (SAH). In the present study, we aimed to synthesize and investigate the cerebroprotective effects of formazans (quinazolinoneformazans – QF_1 and QF_2) for attenuation of subarachnoidhemorrhage induced cerebral vasospasm in rats. Subarachnoid hemorrhage (SAH) was performed by using a blood injection method. The neurological status of all animals was evaluated prior to surgery and 24hr post-surgery before euthanization and brain tissue harvesting. The brains were subjected for histopathological analysis. The cerebral vasospasm was noted measuring the diameter of major carotid artery (MCA) segment and the values from SAH and Sham animals were compared.All animals recovered from surgery and returned to their baseline activity with no motor weakness on spontaneous gait. There was no indication that the procedure caused any significant damage to the brain stem. Histological examination showed normal arterial wall features in control animals. Animals with SAH demonstrated significantly corrugated and thickened wall structure with decreased diameter on cross-section in SAH + vehicle group were significantly different from the sham group. The diameter was attenuated whereas the thickness was increased in the SAH + vehicle group as compared with the sham group. Simvastatin had shown great changes in these parameters while formazans exhibited mild changes in the parameters compared to SAH + Vehicle.From the results obtained the current work demonstrated that the synthesized formazans (QF_1 and QF_2) does not exhibit motor weakness on spontaneous gait. From the histopathological study the formazans have limited significance in attenuation of SAH induced cerebral vasospasm.

Keywords: Subarachnoid Hemorrhage (SAH), Cerebral vasospasm, Simvastatin, Formazans, Major carotid artery

INTRODUCTION

Cerebral vasospasm is delayed-onset cerebral arterial narrowing in response to blood clots left in the subarachnoid space after spontaneous aneurysmal subarachnoid haemorrhage (SAH). High mortality rate due to subarachnoid haemorrhage in humans is mainly due to its complications like cerebral vasospasm, hyponatremia etc. Subarachnoid hemorrhage (SAH) accounts for 5% of strokes **[1]**, it is associated with high mortality and severe morbidity. Although medical technology and treatment modality continues to advance, the prognosis of SAH patients is still poor.Traditionally, delayed cerebral vasospasm, which defined as a pathological demonstration occurred in the late phase of SAH (3-7 days). To date, tremendous research efforts have been made to reduce SAH-induced cerebral vasospasm **[2-4]**. Although positive results were obtained from animal experiments **[5-7]**, however, anti-vasospasm treatments were failed to improve the SAH patient's outcome inmost clinical trials **[8]**. These results from clinical trials make researchers raise doubt on the role of vasospasm,and attempt finding new targets in treating patients suffering from SAH.

Among the wide variety of nitrogen heterocycles that has been explored for developing pharmaceutically important molecules the compounds bearing quinazoline nucleus has wide range of importance in medicinal chemistry, due to wide spectrum of biological activities exhibited by them. Quinazoline formazans have exhibited a variety of biological activities. Literature survey revealed that various substituted quinazolinones are known to possess antimicrobial **[9-11]**, analgesic **[12, 13]**, anti-inflammatory **[14, 15]**, anthelmintic **[16]**, anticonvulsant **[17]**, anti-tubercular **[18]**, anticancer **[19]**, anti-HIV **[20]** properties. All these valid observations led us to investigate some quinazolinone formazan for attenuation of subarachnoid hemorrhage induced cerebral vasospasm in rats.

MATERIALS AND METHODS

Synthesis of formazans:

Two formazans were synthesized viz. 1-(4-Nitrophenyl)-3-(4-chlorophenyl) -4-[benzamido-(2-methyl-3-quinazolin)-4-one]formazan [QF_1] and 1-(3-Fluoro-4-

chlorophenyl)-3-(4-chlorophenyl)-4-[benzamido-(2-methyl-3-quinazolin)-4-one] formazan [QF_2] for the present study by the following steps (Scheme-I) **[21-23]**:

Step-1: Synthesis of 2-methyl-benzoxazin-4-one:

A mixture of equimolar quantities (0.1 mole) 2-amino benzoic acid (anthranilic acid) and acetic anhydride (25 ml) were refluxed for 2hrs and the reaction was monitored by TLC for the completion of reaction. The reaction mixture was poured in ice-cold water. The resulting mass was filtered, dried and recrystallized from DMSO.

Step- 2: Synthesis of 3-(4-carboxyphenyl)-2-methyl-3-quinazolin-4-one:

To a solution of 2-methyl-benzoxazin-4-one (0.01 mole) in alcohol para amino benzoic acid (PABA) was added, refluxed for 4 hrs and the reaction was monitored by TLC for the completion of reaction.

The resulting product is added to ice-cold water, filtered, dried and recrystallized from DMSO.

Step- 3: Synthesis of 3-(4-benzoylchloro)-2-methyl-3-quinazolin-4-one:

To the solution of 3-(4-carboxyphenyl)-2-methyl-3-quinazolin-4-one in alcohol (0.01 mole) double the molar concentration thionyl chloride (0.02 mole) is added. The resulting mixture was concentrated and solid residue formed at the bottom is collected dried and recrystallized from DMSO.

Step- 4: Synthesis of 3-(4-hydrazinobenzoyl)-2-methyl-3-quinazolin-4-one:

To the solution of 3-(4-benzoylchloro)-2-methyl-3-quinazolin-4-one (0.01 mole) in alcohol double the molar concentration (0.02 mole) of hydrazine hydride is added, refluxed for 4 hrs. The resulting product is concentrated and the residue is collected, dried and recrystallized from DMSO.

Step- 5: Synthesis of 3-(4-arylazobenzamido)-2-methyl-3-quinazolin4-one derivatives:

To a solution of 3-(4-hydrazinobenzoyl)-2-methyl-3-quinazolin4-one (0.01 mole) in alcohol, aldehyde or substituted aldehyde (0.01mole) is added, stirred well to get the respective quinazolinone derivatives. The resulting product is filtered, dried and recrystallized.

Step- 6: Synthesis of 1-substituted phenyl-3-substituted phenyl-4-[benzamido (2-methyl-3-quinazolin)-4-one] formazans:

The diazonium salts derived from the respective amines (0.01mole) were added to aryl 3-(4-arylazobenzamido)-2-methyl-3-quinazolin-4-one derivatives in pyridine at 0-5ºC with stirring for 30 minutes and the resultant mixture was added to ice-cold water. The resulting product is filtered, dried and recrystalized from DMSO.

Experimental animals and groups

All experimental protocols were approved by the Institutional animal ethics committee (Reg. No. 1048/PO/Re/S/07/CPCSEA: 18/IAEC/CLPT/2016-17) and were carried out as per the CPCSEA guidelines.

36 Adult male Sprague-Dawley rats (275-300 g) were taken and were randomly divided into four groups: Sham group (sham, n=6);Subarachnoid haemorrhage (SAH) treated with physiological saline as vehicle (SAH +vehicle n=6) ; SAH + QF_1 (n=6); SAH + QF_2 (n=6) and SAH treated with simvastatin which has been previously shown to be neuroprotective and in ameliorating cerebral vasospasm (SAH+ simvastatin, n=6). The neurological status of all animals was evaluated prior to surgery and 24hr post-surgery as earlier reported before euthanization and brain tissue harvesting and the brains were subjected for histopathological analysis.

Induction of SAH [24, 25]:

SAH was performed by using a blood injection method as described previously, with small modification. Animals were anesthetized with halothane (3% induction & 1-2% maintenance). The right femoral artery is exposed. The posterior scalp is incised at the midline and the skull exposed at the junction of the occipital bone. A 30-gauge needle is used to puncture the atlanto-occipital membrane 45° caudally, from the midline toward the left side of the animal, into the cisterna magna. Blood (50–60μl) is withdrawn from the femoral artery and injected through the 30-gaugeneedle into the cisterna magna. The incision closed tightly with sutures to prevent cerebrospinal fluid (CSF) or blood leakage. The leg incision is closed with suture. The peak of the persistent phase of vascular narrowing is between 1 and 3 days after SAH; this is an appropriate time frame for measuring the effects of vascular constriction. All animals were kept in an air-conditioned room with temperature maintained at 27°C, and the rats were allowed access to food and water ad libitum before and after surgery.

Drug administration:

Thirty minutes after the procedure, SAH+simvastatin group received an intra-peritoneal injection of simvastatin (10 mg/kg). Sham + vehicle group was administered with physiological saline. SAH+QF_1 and SAH + QF_2 received an intraperitonial injection of synthetic quinazoline formazans respectively (100 mg/kg).

Neurological evaluation [26]:

Neurological symptoms were evaluated by direct observation of spontaneous activity after the recovery from anesthesia. The evaluation was recorded immediately upon recovery and at 24h postoperatively as either normal activity, weakness on gait with or without generalized weakness.

Assessment of cerebral vasospasm

After 24 hrs, animals were re-anesthetized, were then decapitated and the brains were carefully removed preserving the vasculature. The brains were subjected to histopathological study. The cerebral vasospasm was noted by measuring the diameter of the major carotid artery segment and the values from SAH and sham animals were compared.

Statistical analysis:

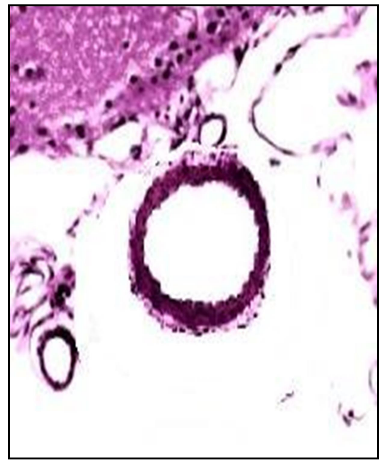
All statistical analysis was performed using Graphpad Prism 5. The data are expressed as mean±S.E.M. Statistical differences between the various groups were assessed with a one-way ANOVA. Differences in the means of arterial diameters were compared by using Dunnett's multiple comparisons test. Because diameters greater than the mean are not physiologically relevant, only the lower tail was considered and were used for vessel diameter comparisons. A value of $p < 0.001$and $p < 0.0001$ *vs* SAH + vehicle was considered statistically significant.

RESULTS

All animals recovered from surgery and returned to their baseline activity with nomotor weakness on spontaneous gait. Gross pathological examination of the meninges and external surface of the brain revealed diffuse blood prominently over the brain stem and the area of the Circle of Willis. There was no indication that the procedure caused any significant damage to the brain stem.

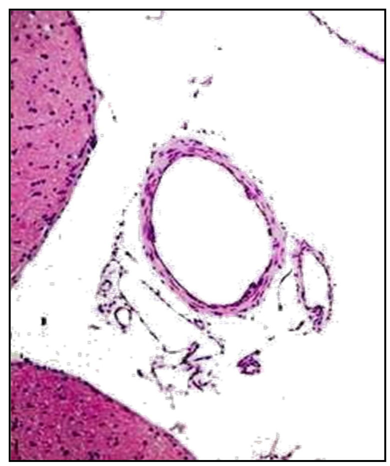
Histological examination showed normal arterial wall features in control animals. Animals with SAH demonstrated significantly corrugated and thickened wall structure with decreased diameter on cross-section **(Figure 1)** in SAH + vehicle group were significantly different from the sham group. The diameter was attenuated whereas the thickness was increased in the SAH + vehicle group (214.8 ± 7.44μm) as compared with the sham group (259.7 ± 6.46μm). Simvastatin had shown great changes in these parameters(262.8 ± 5.87; $p < 0.0001$ vs. SAH+Vehicle) while SAH + QF_1 andSAH + QF_2 groups (treated with the formazans) moderately differ in the parameter compared with the SAH group (232.2 ± 4.47 μm; 229.8 ± 5.05 μm, $p<0.001$ respectively) **(Table 1, Figure 2)**.

Sham



SAH+Vehicle

SAH+Simvastatin



SAH+QF_2

Figure 1: Quantification of Vasospasm in Thickness and diameter (µm)

Table 1: Quantification of vasospasm (MCA diameter)

Treatment	MAC Diameter (µm)
Sham	259.7 ± 6.46
SAH + Vehicle	214.8 ± 7.44
SAH + Simvastatin	262.8 ± 5.87****
SAH + QF_1	232.2 ± 4.47***
SAH + QF_2	229.8 ± 5.05***

Note: Each value represents the mean ± SEM (n = 6). ***p<0.001 and ****p<0.0001 compared with SAH + Vehicle group

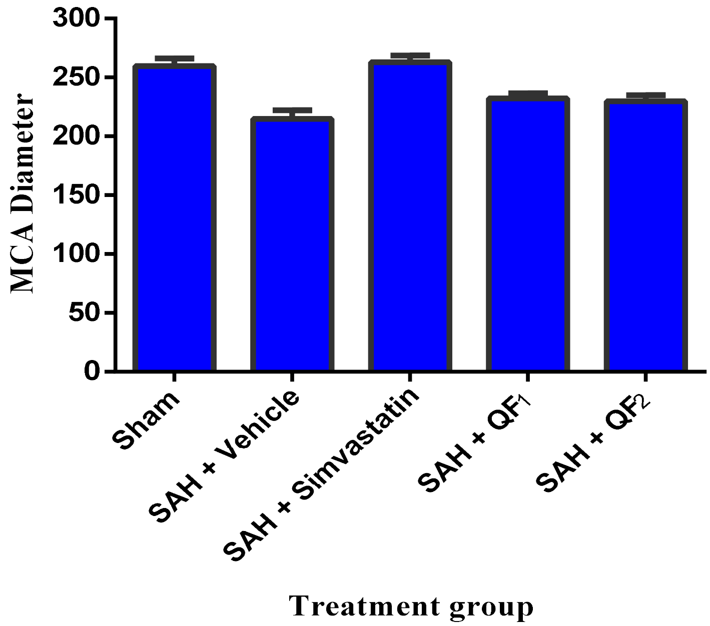


Figure 2: Quantification of vasospasm (MCA diameter)

DISCUSSION

Statins, are widely used clinically for their lipid-lowering properties **[27]**, recent evidence shows that statins are also effective in ameliorating cerebral vasospasm, which occurs as a sequel of SAH **[28]**. Two randomized clinical trials investigating the use of statins for vasospasm after aneurysmal SAH showed that acute treatment with statins after SAH is safe and ameliorates vasospasm **[29]**. In the present study simvastatin significantly reversed the decrease in arterial diameter as well as the vessel wall thickening observed in the MCA affected by cerebral vasospasm at 24 hrs after SAH. Both Formazans (QF_1 and QF_2) moderately influence the cerebral vasospasm as indicated by morphological data (compared with sham animals).

CONCLUSION

From the results obtained the current work demonstrated that the synthesized formazans (QF_1 and QF_2) does not exhibit motor weakness on spontaneous gait. From the histopathological study the formazans have limited significance in attenuation of SAH induced cerebral vasospasm.

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CONFLICTS OF INTEREST

There are no Conflict of Interest.

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