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EFFECT OF SALIVARY GLAND EXTRACT OF *ARIOPHANTA PULMONATA* ON ANGIOGENESIS OF TERTIARY VITELLINE VEINS IN CHICK EMBRYO

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ABSTRACT
Angiogenesis is the formation of new blood vessels from existing vessels. Natural inhibitor blocking the formation of new blood vessels is a promising anti cancer strategy or even in wound healing process. Chorioallantoic membrane (CAM) is well vascularized and used to study as in vivo model of angiogenesis. In the present investigation effect of salivary gland, alcohol extract of *Ariophanta pulmonata* on angiogenesis especially tertiary vitelline veins was undertaken. Fertilized eggs of *Gallus gallus* were incubated at 38ºC and 70% relative humidity. In the embryos of 48, 55, 66, 72, 88 and 96 hrs CAM was exposed to 1 mg/ml salivary gland extract and were further incubated up to 144 hrs and CAM was studied. Results of the present study indicate significant inhibitory effect of the salivary gland extract on the number and area of tertiary vitelline veins (TVV). The maximum inhibition in number and area of TVV was found when the embryos were treated at 48 hrs and showed 70.70% and 80.32% depletion in the number and area of TVV respectively. Inhibition of angiogenesis was highly significant at 48, 55 and 66 hrs as compared to 72, 88, 96 hrs of incubated chick embryos. The antiangiogenic role of complex carbohydrates is discussed.

INTRODUCTION

The process of angiogenesis is the formation of new blood vessels from existing vessels. Angiogenesis occurs in response to specific signals and the extra-cellular matrix of the cell. Angiogenesis is not only involved in cancer development but also in many diseases. A number of natural inhibitors have been identified that can block the formation of new blood vessels. This idea is now blossomed into very promising anticancer strategy or even in wound healing process. After reviewing the literature anti-angiogenesis role of glycosaminoglycans (Hahnenberger *et al.*, 1991) and anti-proliferative and apoptotic potential of tick salivary gland was observed. (Kazimirova, 2006). Hence it was decided to undertake the present investigation on effects salivary gland extracts of *Ariophanta pulmonata* on angiogenesis.

Chorioallantoic membrane (CAM) of chick embryo performs functions as gas exchange and waste elimination between embryo and outside which influences the growth of embryo (Patten, 1977; Carlson, 2007). It is being well vascularised and is used to study *in vivo* model of angiogenesis (Krunzi-Rapp, 2003) to evaluate proangiogenic and antiangiogenic potency of substances (Wilting *et al.,* 1991, Ribatti *et al.,* 1999). The present communication deals with angiogenic properties of salivary gland extracts of *Ariophanta pulmonata*.

MATERIALS AND METHODS

Fertilized eggs of Gallus gallus was selected for the present investigation. They were incubated at temperature 38ºC and relative humidity of 70%.

The incubation was conducted to obtain embryos of 48, 55, 66, 72, 88, 96 hrs. On completion of experiment from each of the embryos CAM was exposed to 1mg/mL salivary gland alcohol extract as described in Table 1 by window method (Korn and Cramer, 2007). The embryos were further incubated up to 144 hrs and CAM was studied. Statistical analysis was done as per (Fisher and Yates, 1938). In progressive growth of CAM during development at 48 hrs, right and left primary vitelline veins are formed, at 55 hrs from these veins secondary vitelline veins are initiated, at 66 hrs posterior vitelline vein appears, at 72 hrs terminal capillaries or tertiary vitelline veins (TVV) are networked, at 96 hrs all these vitelline veins and their capillaries are well defined.

Extract preparation

For the dose administration Hanks Balanced Salt Solution (HBBS) is used as saline. Salivary gland extract was prepared in alcohol. 20 mg of salivary gland of *A. pulmonata* was homogenated in 1 mL of distilled water. Then 5 mL alcohol is added in the homogenate and kept it at 10ºC for 5 to 7 hrs., centrifuged and evaporated the supernatant to remove alcohol and dissolved the residue as per the dose in HBSS (Table 1).

RESULTS

Results have been presented in Table 2 and figures 1,2, 3 and 4.

Tertiary vitelline veins in embryos treated at 48 hrs

Table 1: Groups as per treatment and development

Groups as per Treatment	Groups as per developmental stages in hrs.	Groups as per time of exposure initiations 48	55	72	78	89	96	Final developmentIn hr and hr of sacrifice
Group I Normal	Group I maintained for all the hrs independently	-	-	-	-	-	-	Animals from 48,55,66, 72,88,96 hrs belonging to Groups I to III were sacrificed after 144 Hrs
Group II HBSS	Group II for 48,55, 66, 72,88, 96hrs	✓	✓	✓	✓	✓	✓	
Group III 1mg/ml salivary gland alcohol extract	Group III for 48,55,66, 72, 88,96hrs	✓	✓	✓	✓	✓	✓	

Right side: In normal embryos the number of tertiary vitelline veins was 92 and the area covered by them was 0.51sq.mm. HBSS treatment to embryos decreased the number of the vitelline veins by 8.61% but area covered by them was increased by 1.60 fold as compared to normal. Treatment of 1 mg/mL salivary gland alcohol extract to normal embryos showed depletion in number of TVV by 62.96% and area covered was decreased by 65.69%. When compared these results with that of HBSS treated control embryos the number TVV was decreased by 59.83% and 79.05% depletion in area covered by them (Table 2).

Left side: In normal embryos the member of TVV was 99 and area covered was 0.58 sq.mm. HBSS treatment to the embryos did not change the number of TVV but area associated with them was increased by 1.12 fold. Treatment of 1 mg/mL salivary gland alcohol extract to normal embryos resulted in 70.70% depletion in number of TVV and 80.32% depletion in area covered by them. As compared to HBSS treated control embryos showed 82.54% depletion in the area associated with TVV.

Tertiary vitelline veins in embryos treated at 55 hrs

Right side: In normal embryos 100 TVV were observed area covered by them was 0.51 sq.mm. HBSS treatment to the normal embryos increased by 1.01 fold and covered area was increased by 1.09 fold. Administration of 1 mg/mL salivary gland alcohol extract to normal embryos showed 72% decrease in number of TVV and 67.67% depletion in area associated with them. As compared to HBSS treated control embryos area was decreased by 70.65% (Table 2).

Left side: The number of TVV was 83 and the area covered by them was 0.56 sq.mm. HBSS treatment to embryos showed increase in the number of TVV by 1.21 fold and area was marginally increased. Treatment of 1 mg/mL salivary gland alcohol extract to normal embryos showed 69.68% depletion in the number of TVV and 86.75% depletion in area covered by them. As compared to HBSS treated control embryos number to TVV was depleted by 75.26%.

Tertiary vitelline veins in embryos treated at 66 hrs

Right Side: In normal embryos TVV were 96 and area covered was 0.65 sq.mm. HBSS treatment to the embryos showed 1.07 fold increase in number of TVV and area covered by them was marginally increased. Treatment of 1 mg/mL salivary gland alcohol extract to normal embryos showed 43.71% depletion in number of TVV and 68.70% depletion in area covered by them. As compared to HBSS treated control embryos number of TVV was decreased by 46.55% (Table 2).

Left side: At 66 hrs the number of TVV was 85 and associated area was 0.50 sq.mm. HBSS treatment to the embryos showed increase in number of TVV by 1.31 fold and area was increased by 1.14 fold. Administration of 1 mg/mL salivary gland alcohol extract to normal embryos showed 39.83% depletion in number of TVV and area covered by them was decreased by 48%. Comparison of these that of HBSS control embryos showed 54.58% depletion in number of TVV and 55.39%

Table 2: Effect of salivary gland alcohol extract of *A. pulmonata* on tertiary vitelline veins in CAM of chick embryos

Groups	Inc.hrs.	Right no	Sq. area cm	Left no	Sq. area cm	Total Total no.	Total area
Normal	48	92 ± 5.4	0.51 ± 0.026	99 ± 1.73	0.58 ± 0.022	191 ± 6.29	1.09 ± 0.039
	55	100 ± 2.91	0.51 ± 0.015	83 ± 3.13	0.56 ± 0.021	183 ± 5.97	1.07 ± 0.031
	66	96 ± 1.93	0.65 ± 0.021	85 ± 2.15	0.50 ± 0.023	181 ± 3.87	1.15 ± 0.039
	72	97 ± 6.06	0.59 ± 0021.	79 ± 1.95	0.51 ± 0.013	176 ± 7.87	1.10 ± 0.029
	88	97 ± 5.26	0.53 ± 0.015	69 ± 2.74	0.56 ± 0.021	166 ± 7.57	1.09 ± 0.038
	96	105 ± 1.65	0.56 ± 0.021	66 ± 2.69	0.49 ± 0.034	171 ± 4.37	1.05 ± 0.047
HBSS Control	48	85 ± 2.34	0.82 ± 0.045	99 ± 2.11	0.65 ± 0.024	184 ± 3.89	1.47 ± 0.064
	55	101 ± 1.87	0.56 ± 0.023	101 ± 1.85	0.58 ± 0.018	202 ± 3.49	1.14 ± 0.037
	66	103 ± 2.08	0.69 ± 0.017	112 ± 4.16	0.57 ± 0.028	215 ± 5.97	1.28 ± 0.034
	72	95 ± 2.22	0.88 ± 0.025	102 ± 1.37	0.71 ± 0.012	197 ± 3.54	1.59 ± 0.031
	88	104 ± 6.5	0.52 ± 0.028	83 ± 1.97	0.61 ± 0.013	187 ± 7.87	0.59 ± 0.027
	96	109 ± 1.06	0.69 ± 0.037	71 ± 4.18	0.58 ± 0.019	180 ± 5.11	1.27 ± 0.051
1mg/mL salivary	48	35 ± 2.38^{cz}	0.18 ± 0.023^{cz}	30 ± 1.66^{cz}	0.12 ± 0.010^{cz}	65 ± 3.75^{cz}	0.30 ± 0.029^{cz}
gland alcohol	55	28 ± 1.74^{cz}	0.17 ± 0.023^{cz}	26 ± 1.43^{cz}	0.091 ± 0.022^{cz}	54 ± 3.05^{cz}	0.26 ± 0.039^{cz}
extract	66	55 ± 2.74^{cz}	0.21 ± 0.015^{cz}	52 ± 2.67^{cz}	0.26 ± 0.022^{cz}	107 ± 3.38^{cz}	0.47 ± 0.033^{cz}
	72	42 ± 2.01^{cz}	0.23 ± 0.018^{cz}	47 ± 1.07^{cz}	0.27 ± 0.014^{cz}	89 ± 3.03^{cz}	0.50 ± 0.029^{cz}
	88	47 ± 1.71^{cz}	0.25 ± 0.014^{cz}	39 ± 2.51^{cz}	0.28 ± 0.014^{cz}	86 ± 3.89^{cz}	0.53 ± 0.025^{c}
	96	43 ± 2.54^{cz}	0.31 ± 0.020^{cz}	27 ± 2.47^{cz}	0.32 ± 0.027	70 ± 4.73^{cz}	0.63 ± 0.045^{cz}

Results expressed as mean ± S.E. of 5 embroys; P. values a < 0.05, b < 0.01, c < 0.001 vs Normal embryos; x < 0.05, y < 0.01, z < 0.001 vs HBSS control embryos

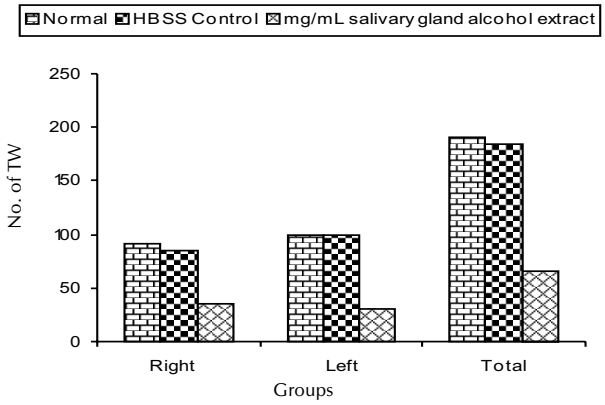

Figure 1a: Effect of salivary gland extract on tertiary vitelline veins (Gr. I, 48+96 = 144 hr)

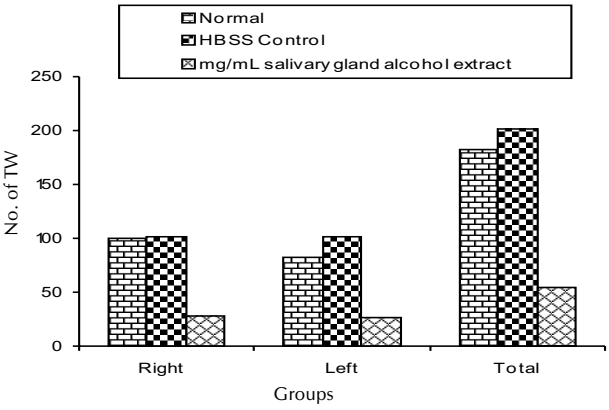

Figure 1b: Effect of salivary gland extract on tertiary vitelline veins (Gr. II, 55+89 = 144 hr)

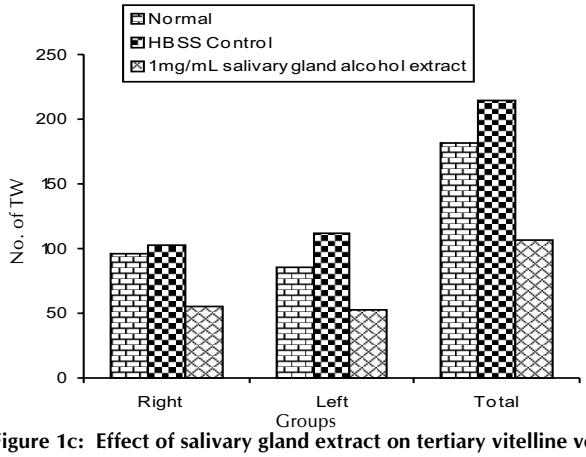

Figure 1c: Effect of salivary gland extract on tertiary vitelline veins (Gr. III, 66+78 = 144 hr)

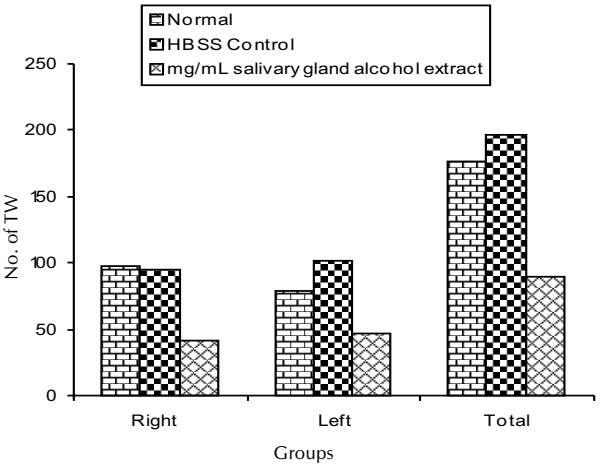

Figure 1d: Effect of salivary gland extract on tertiary vitelline veins (Gr. IV, 72+72 = 144 hr)

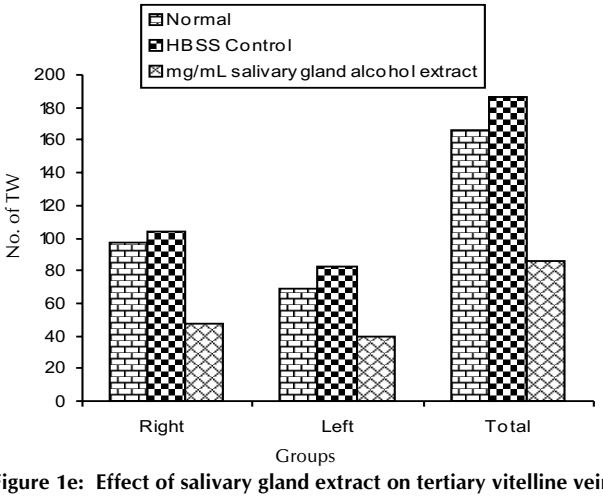

Figure 1e: Effect of salivary gland extract on tertiary vitelline veins (Gr. V, 88+56 = 144 hr)

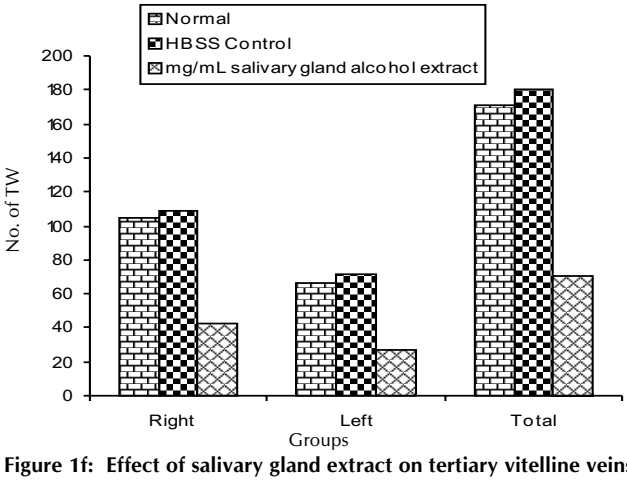

Figure 1f: Effect of salivary gland extract on tertiary vitelline veins (Gr. VI, 96+48 = 144 hr)

depletion in area covered by them.

Tertiary vitelline veins in embryos treated at 72 hrs

Right Side: In normal embryos TVV were 97 and area covered was 0.59 sq.mm. HBSS treatment to embryos showed marginally increased number of TVV but area covered by them was increased by 1.49 fold treatment of 1 mg/mL salivary gland alcohol extract to embryos showed 57.71% depletion in number of TVV and 62.02% depletion in area covered by them. As compared to HBSS treated control embryos area covered by them was depleted by 74.87% (Table 2).

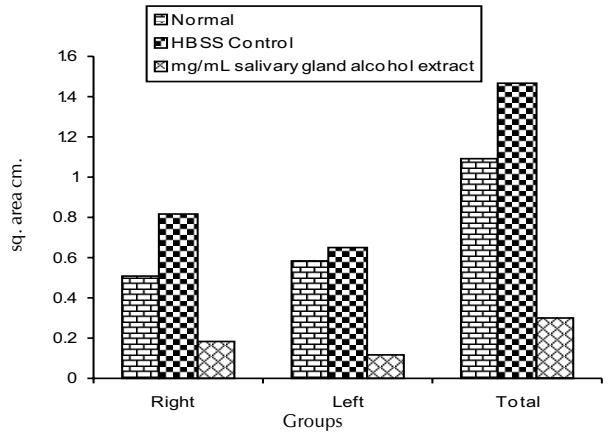

Figure 2a: Effect of salivary gland extract on tertiary vitelline veins (Gr. I, 48+96 = 144 hr)

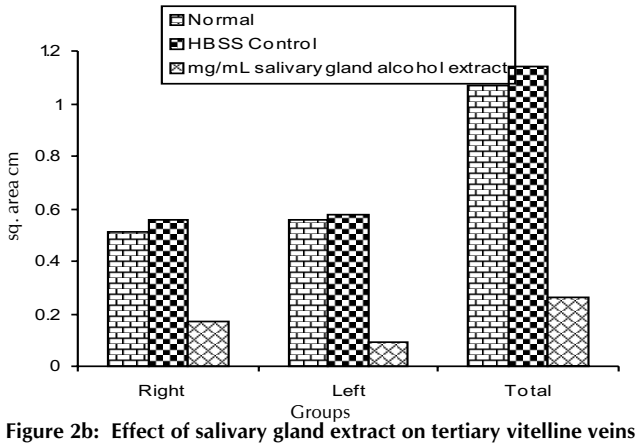

Figure 2b: Effect of salivary gland extract on tertiary vitelline veins (Gr. II, 55+89 = 144 hr)

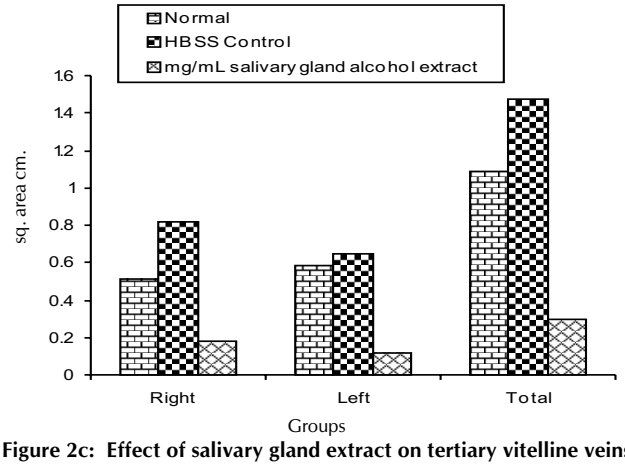

Figure 2c: Effect of salivary gland extract on tertiary vitelline veins (Gr. III, 66+78 = 144 hr)

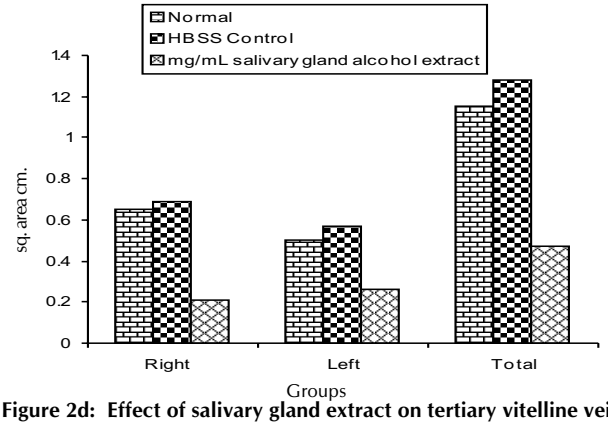

Figure 2d: Effect of salivary gland extract on tertiary vitelline veins (Gr. IV, 72+72 = 144 hr)

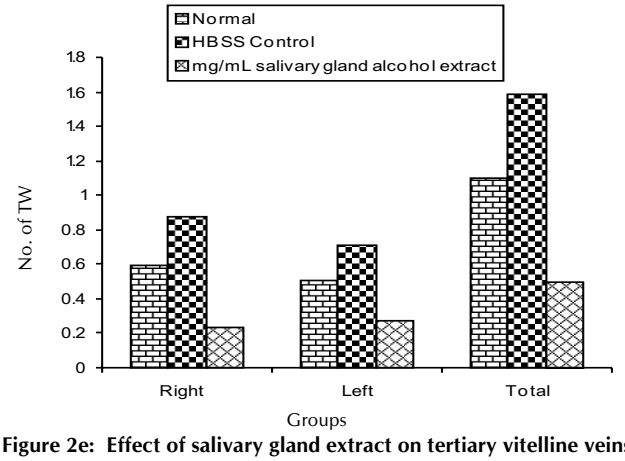

Figure 2e: Effect of salivary gland extract on tertiary vitelline veins (Gr. V, 88+56 = 144 hr)

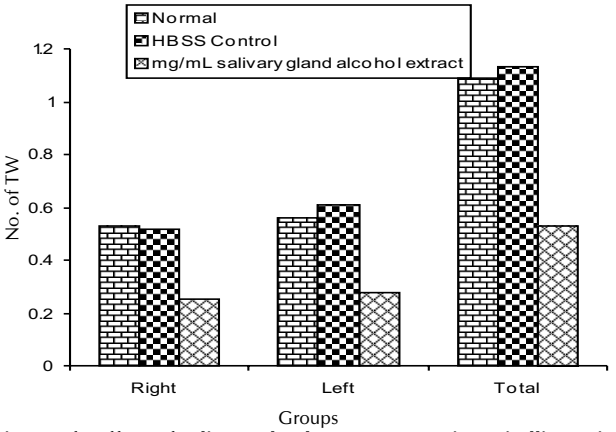

Figure 2f: Effect of salivary gland extract on tertiary vitelline veins (Gr. VI, 96+48 = 144 hr)

Left Side: At 72 hrs the number of TVV was 79 and associated .area was 0.51sq.mm. HBSS treatment to embryos showed increase in the number of TVV by 1.29 fold and associated area was increased by 1.39 fold as compared to normal. Treatment of 1 mg/mL salivary gland alcohol extract to embryos resulted in 41.51% depletion in number of TVV and area associated with them was depleted by 62.98%. While comparison with HBSS treated control embryos number of TVV was depleted by 54.93% and 62.98% depletion in area covered by them.

Tertiary vitelline veins in embryos treated at 88 hrs

Right side: Normal embryos were with 97 TVV and associated

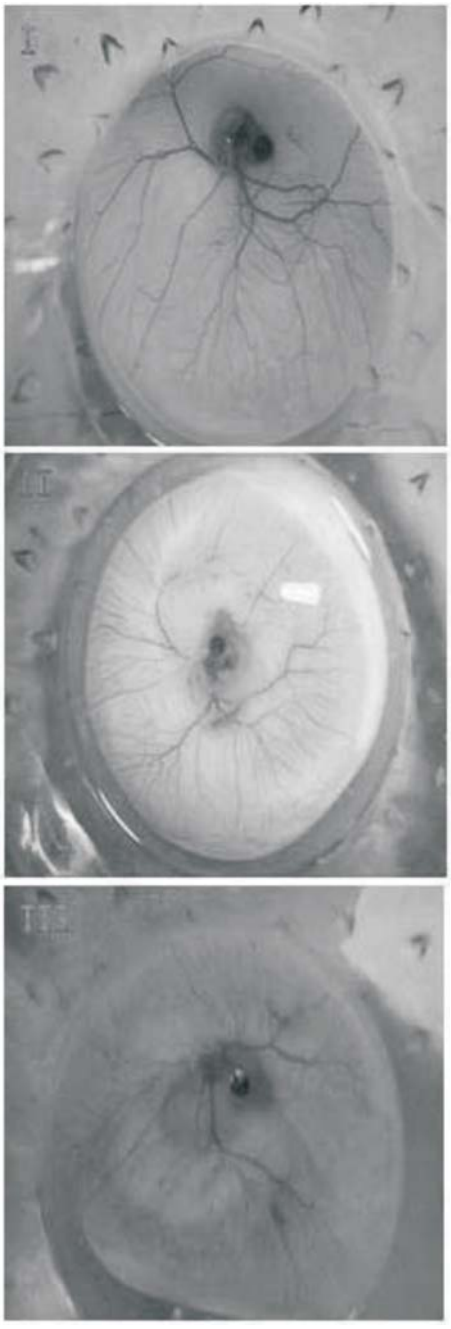

Figure 3 (I to III in 48 hrs Set): Showing alterations by salivary gland alcohol extract in the angiogenesis of CAM of Chick embryo. Dose initiation at 48 hr and after final development of 144 hr; (I) Normal: Normal Angiogenesis in CAM of chick embryo after 144 hr of development; (II) Control (HBSS): Figure shows increase in the angiogenesis than the normal and increase in the area covered by the tertiary vitelline veins than normal; (III) 1 mg/mL salivary gland alcohol extract: Figure shows reduction in the normal angiogenesis of CAM than normal and also reduction in number and area covered by the vitelline veins.

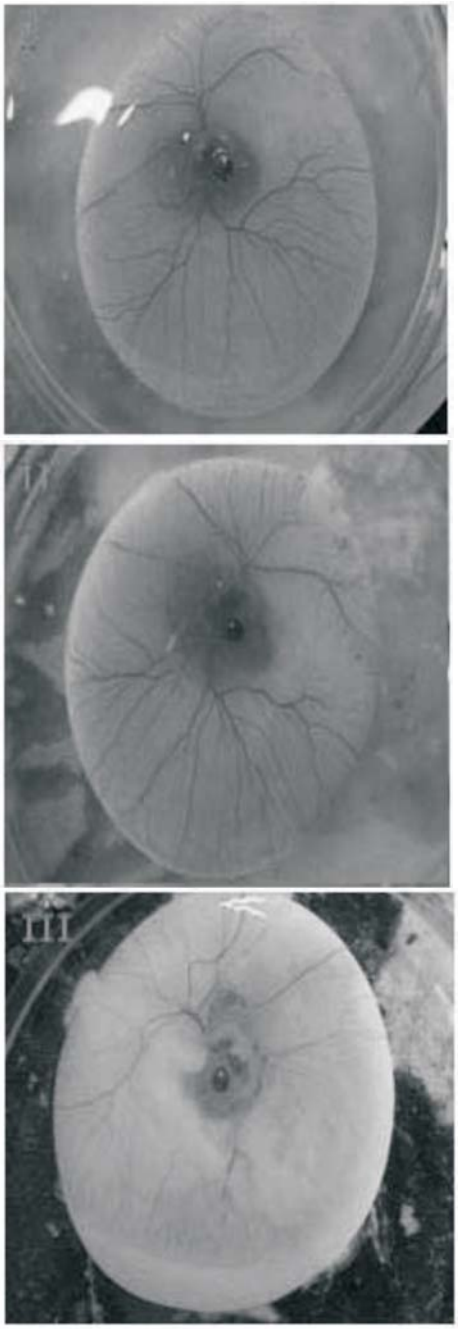

Figure 4 (I to III in 72 hrs Set): Showing alterations by salivary gland alcohol extract in the angiogenesis of CAM of Chick embryo. Dose initiation at 72 hr and after final development of 144 hr; (I) Normal: Normal Angiogenesis in CAM of chick embryo after 144 hr of development; (II) Control (HBSS): Figure shows increase in the angiogenesis than the normal and increase in the area covered by the tertiary vitelline veins than normal; (III) 1 mg/ml salivary gland alcohol extract: Figure shows reduction in the normal angiogenesis of CAM and also reduction in number and area covered by the vitelline veins

area was 0.51 sq.mm. HBSS treatment to the normal embryos showed 1.07 fold increase in number of TVV and area associated with them was insignificantly decreased. Administration of 1 mg/mL salivary gland of 1 mg/ml salivary gland alcohol extract to embryos showed 52.55% depletion in number of TVV and area covered by them was depleted by

53.84%. Comparison of results that of HBSS treated control embryos showed 55.81% depletion in number of TVV (Table 2).

Left Side: In normal embryos 69 TVV were observed with area covered 0.56 sq.mm. HBSS treatment increased the number of TVV by 1.20 fold and associated area was increased by 1.08 fold. Treatment of 1 mg/ml salivary gland alcohol extract showed 44.48% depletion number of TVV and 50% depletion in area covered by them. As compared to HBSS treated HBSS treated control embryos showed 54.02% depletion in number of TVV and 55.10% decrease in area covered by them.

Tertiary vitelline veins in embryos treated at 96 hrs

Right Side: At 96 hrs the normal embryos showed 105 number of TVV and the area covered was 0.58 sq.mm. HBSS treatment to the embryos showed increase in TVV by 1.03 fold and area associated with them was increased by 1.18 fold. Administration of 1 mg/mL salivary gland alcohol extract to normal embryos showed 60.05% decrease in number of TVV and 47.56% depletion in area covered by them. As compared to HBSS treated control embryos showed 61.56% decrease in number of TVV and 56.08% depletion in area covered by them (Table 2).

Left Side: In normal embryos the number of TVV was 66 and associated area was 0.49 sq.m. HBSS treatment to the normal embryos resulted in 1.07 fold increase in number of TVV and 1.18 fold increase in area associated with them. Treatment of 1 mg/mL salivary gland alcohol extract to normal embryos showed 60.10% decrease in number of TVV and 35.70% depletion in area covered by them. As compared to HBSS treated embryos showed 562.98% decrease in number of TVV and 45.83% depletion in area covered by them.

DISCUSSION

In the entire experiments it is observed that tertiary vitelline veins are most affected and show maximum inhibition. Maximum number and area of TVV was found to be inhibited when the embryos were treated at 48 hrs. with salivary gland extract in alcohol. The embryos treated with salivary gland alcohol extract showed 70.70% and 80.32% depletion in the number and area of TVV respectively. Salivary glands had inhibitory effect on angiogenesis. The inhibitory effect of sulphated and non sulphated glycosamino glycans on the normal outgrowth of capillaries was tested in the chick embryo chorioallantoic membrane (CAM). The result showed that sulphation of glycosaminoglycans and polysaccharides increases or induces an antiangiogenic effect (Hahnenberger and Jakobson, 1991). A potent angiogenesis inhibitor u-995 has been purified from the cartilage of the blue shark *Prionace gluca* (Sheu *et al.,* 1998). Above research indicate that sulphated and non sulphated mucopolysaccharides had an antiangiogenesis effect. Salivary glands contain mucins in greater proportion. Some of these complex carbohydrate might be playing a role in antiangiogenesis. More elaborate studies will be helpful to isolate and identify antiangiogenic factor from animal origin

In conclusion angiogenesis is influenced by salivary gland extract of *A. pulmonata*. Decrease in angiogenesis was highly significant evidently at 48, 55 and 72 hrs as compared to 72, 88 and 96 hrs. of incubated chick embryos.

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