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REPURPOSING OF ANTI-MALARIAL SYNRIAM™ AND TESTING ITS EFFICACY AGAINST EGYPTIAN STRAIN OF *SCHISTOSOMA HAEMATOBIUM*

By

HEBAT-ALLAH S.A. YOUSOF[1*], SHAIMAA H. EL-SAYED[2], EMAN ELFAR[3], and MONA M. KHATER[1]

Department of Medical Parasitology[1], Faculty of Medicine, Cairo University, Cairo, Department of Medical Parasitology[2], Faculty of Medicine, Helwan University, Cairo, and Department of Public Health and Community Medicine[3], Faculty of Medicine, Cairo University, Cairo, Egypt.

(*Correspondence: drhebasalah@kasralainy.edu.eg

-ORCID: https://orcid.org/0000-0001-7214-0991)

Abstract

Trials for discovering an anti-malarial drug, which can compete Schistosomal infection in co-endemic areas, are ongoing. Some preliminary studies were done on Synriam (SYN), anti-malarial drug combination (arterolane maleate and piperaquine phosphate) released from Ranbaxy, to test its anti-schistosomal effect. But, in vitro incubation of SYN with different *Schistosoma haematobium* stages was not fully assessed. This study determined the anti-schistosomal in vitro effect of SYN on adult and juvenile stages of *Schistosoma haematobium*-Egyptian strain. Adult and juvenile worms were incubated with ascending concentrations of SYN and with praziquantel as positive control. Viability, survival, morphological and ultra-structural changes were assessed at different time points. Higher concentrations of 60, 80 µg/ml showed rapid and lethal effects on adult and juvenile stages of both species, with prominent ultrastructural alterations. Concentrations of (10, 20, & 40µg/ml) showed mild to moderate effect on adult schistosomes. On contrarily to praziquantel, larval immature stages responded significantly and rapidly to low concentration of SYN with 100% death rate. The present findings were consistent with the evaluation of anti-schistosomal therapeutic effect of SYN to be utilized in malaria co-endemic areas.

Key words: Anti-schistosomal, *In-vitro*; *Schistosoma haematobium; Schistosoma mansoni*; Synriam; Drug repurposing.

Introduction

Schistosomiasis is ranked as the second most prevalent devastating tropical disease in Africa after malaria. It affects over 250 million people worldwide (Gray *et al,* 2010). Digenetic worms of the genus *Schistosoma*; *Schistosoma mansoni (S. mansoni), Schistosoma haematobium (S. haematobium),* and *Schistosoma japonicum (S. japonicum)* are incriminated in most of human schistosomiasis infections. Control programs were faced by clinical emergence of praziquantel-resistant strains and praziquantel therapeutic deficient activity against immature juvenile stages (Utzinger *et al,* 2003; Ho *et al,* 2014). Accordingly, there was an urgent need to discover and develop new better acting drugs.

Within the same context, being the current treatment of choice for resistant strains of malaria (Giao *et al,* 2001), Artemisinin derivatives based on trioxane (ARTs), have shown reliable in vitro and in vivo anti-schistosomal effect, especially on immature juvenile stages (Bartley *et al,* 2008; Boissier *et al,* 2009; Keiser *et al,* 2012). Moreover, the trioxolanes, OZ78, OZ277 and OZ209 in particular, showed potent activity against juvenile *S. mansoni* and *S. japonicum* infections in the mouse model (Xiao *et al,* 2007). In 2011, after Ranbaxy Laboratories Limited (India) has licensed Synriam™ (SYN)-arterolane maleate (OZ277) and piperaquine-, to be used as a treatment for malaria patients. Mossallam *et al.* (2015) proved the powerful activity of this synthetic derivative in mice infected with *S. mansoni* especially against the parasite juvenile stages. Working

on the promising use of a single effective drug against both blood parasites in co-endemic areas is imperative.

In the framework of Synriam testing, this aimed to investigate the *in-vitro* schistosomicidal properties of Synriam using multiple ascending concentrations against *S. haematobium* developmental stages using light and scanning electron microscopic (SEM) observations.

Material and Methods

Animals and parasites: Syrian golden hamsters (*Mesocricetus auratus*) of both sexes, aged 4–6 weeks were purchased form the Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute (TBRI), Giza, Egypt, and kept under favorable conditions of 25˚C temperature, 70% humidity, 12-hr light and 12-hr dark cycle and adapted for 1 week before infection. Animals were infected percutaneously with freshly shed Egyptian strain of *S. haematobium* cercariae from experimentally infected *Bulinus trucatus* snails (De Souza *et al,* 1979).

Early stages Schistosomulae were obtained by mechanical transformation of *S. haematobium* cercariae (Keiser, 2009; Manneck *et al,* 2009). While that of hepatic stages were recovered by portal-mesenteric perfusion at the 42nd day post infection. Also, adult *S. haematobium* worms were recovered by perfusion of vesical venous plexus at the 90th day post infection (Duvall and Dewitt, 1967).

Drug and reagents: Synriam^{TM} (Arterolane maleate, 150mg and Piperaquine phosphate, 750mg) obtained from Ranbaxy Laboratory Limited, India. Praziquantel (PZQ) tablets (Distocide®) purchased from EIPICO (Cairo, Egypt). Chemicals and solvents were delivered by Sigma, St. Louis, USA. RPMI 1640 medium supplemented with 2mM of L-glutamine, 25 mM Hydroxyethyl-piperazine-ethanesulfonic acid, 20% foetal calf serum, 300µg/ml streptomycin, 160µg/ml gentamycin and 300IU/ml penicillin were used for the *in vitro* culture (Synder *et al,* 2006). Synriam was dissolved in dimethyl sulfoxide (DMSO) (10mg/ml), and then diluted in the culture medium.

Preparation of parasites and experimental design for *in vitro* culturing: Recovered juvenile and adult (male and female) worms were washed 10 times in RPMI medium, taking great care for parasite tegument integrity. Worms were placed in wells of sterile, flat-bottom, 24-well plates (Corning, NY) containing 2ml RPMI 1640 medium /well and incubated at 37°C and 5% CO2 atmosphere (Mossallam *et al,* 2015). To mimic an in-vivo physiological environment, serum albumin (SA) and human alpha acidic glycoprotein (AGP) were added to the culture medium (Beckmann *et al,* 2014).

Before beginning the experiment, worms were being assessed hourly, using light microscope (Olympus Inverted Microscope Model IX70; Olympus, Tokyo, Japan). Only viable, transparent, contractile worms showed total tegument integrity were included in the experiment while parasites that were contracted or had acquired an opaque appearance were considered dead and discarded.

The crowds of worms (adult stage, early and late juvenile stages) were grouped as follows; Experiment I: adult *S. haematobium* (90days), experiment II: early schistosomula *S. haematobium* (3-hours), experiment III: late schistosomula *S. haematobium* (42 days).

Each experiment was tested as 3 groups (12/well); Ga: Medium +0.5% DMSO (negative control), Gb: Medium +1µg/ml PZQ (positive control) and Gc: medium +SYN (test group). Gc was further subdivided into subgroups by using different concentrations of the test drug (SYN was used to obtain final concentrations of 5 to 80µg/ml (5, 10, 20, 40, 60, & 80µg/ml]).

In each experiment, the survival was monitored in (1st, 3rd, 24th & 48th hours) post-incubation with SYN, for body motility and occurrence of death using the dissecting microscope. Worms showed no body movement for at least 30 seconds observation was considered as dead (Xiao *et al,* 2007). All

tests were repeated three times, viability data from one experiment was presented as range of scores (0-3) based on the motility of the worms; 0 (all worms are dead), 1 (minimal motor activity), 2 (slow motor activity) and 3 (normal motor activity) (Lali *et al,* 2015; Guidi *et al,* 2016). Mean ±SD were determined for each independent experiment over the groups.

Ultrastructural studies using SEM: Ultrastructural features of samples of juvenile and adult Shistosomes were examined and compared to control groups for the effect of the tested drugs. Worms were washed out in PBS from the culture solution, fixed in Karnovsky's solution, for 10hours (hrs), and then cleared by keeping them overnight at 4˚C in PBS. The samples were immediately processed (Glauert, 1974). The samples were fixed in equal volumes of glutaraldehyde 4% + cacodylate 0.2 % for 2hrs, washed in equal volumes of sucrose 0.4% and cacodylate 0. % for 2hrs followed by fixation in equal volumes of osmic acid 2% and cacodylate 0.3% for 1hr and then washed with distilled water. Finally, samples were dehydrated in ascending grades of ethanol for 5 minutes (min) each (30%, 50%, 70% & 90%) then 100% absolute ethanol for 10 min. thrice. Examination was done using Environmental SEM (Inspect S; FEI, Holland) at Electron Microscopy Unit, TBRI.

Ethical consideration: The protocol of this study was approved by scientific research ethical committee of TBRI, Giza, Egypt. All animal experiments were performed in accordance with the Egyptian National Animal Welfare Standards and under recommendations of the TBRI Ethical Committee for laboratory animal research guidelines.

Statistical analysis: Data was entered and analyzed using SPSS Version 21 (IBM Corporation, NY, USA). Survival analysis was done using the Kaplan-Meier method. The log-rank test was used for pairwise comparison between different groups in each experiment. P value less than or equal to 0.05 was considered significant. Viability scoring was calculated using Kruskal-Wallis Test (non-parametric test) and displayed as Viability score box-plot. Viability data from one experiment having average replicate was presented as range of scores. Mean values were determined for each independent experiment over the group. The mean ± SD of three viability mean values (of three independent experiments) for each group was calculated at different time points and used to examine if differences between the groups were statistically significant.

Results

Assessment of survival and viability of adult and juvenile stages of *S. haematobium* during in vitro incubation with different concentrations (conc.) of SYN were given (Fig. 1).

Adult *S. haematobium* stage: Similar to control group, incubation with 5µg/ml revealed normal activity (motility score: 3) and outline till the second day of incubation. Diminished motility and abnormal body attitude were observed in a gradual progressive manner, starting form 1 hr and 3 hrs of incubation with 40µg/ml, 20µg/ml, respectively. Stretching and thinning of the whole worm up to complete deformed outline and loss of sucking ability, as evidenced by detachment of worms from sides of wells, were significant with higher concentrations (60µg/ml, 80µg/ml), since the 1sthr of incubation (P <0.005). Motility and viability of worms was affected significantly according to dose of SYN and time of exposure (P <0.001). Mortality was reported initially using 40µg/ml (16.7%) and reached 75% after 24hrs. Meanwhile, death rate was 91.7% & 100% when adult worms were incubated with 60-µg/ml & 80µg/ml, respectively. All worms were dead by the end of experiment upon incubation with all concentrations used.

Juvenile *S. haematobium* stages: Immature stages were susceptible to lower concentrations of SYN. Normal body movement (motility score: 3) was noticed after 3hrs of incubation with 5 µg/ml. In both early and late stages, reduced motility and body stiffness

was significant at conc. of 10µg/ml within 1st hr, which increased progressively with incubation duration. Unlike the adult, 100% death rate was reached after 24hrs of incubation starting from dose of 20 µg/ml. Interestingly, Kaplan-Meier survival analysis revealed significant effect on 3-hour and 42-days aged schistosomula stages using 40µ / ml & 20µg/ml, respectively, in comparison to PZQ-treated groups (P <0.001).

Adult *S. haematobium* stage: SEM micrographs revealed normal appearance of tegument and suckers (Fig. 3A) using low concentrations (5 &10µg/ml) at beginning of the experiment, followed by slight puffiness of the oral sucker and scattered vesicles by the end of the first day (Fig. 3B). Upon early exposure to 20 & 40µg/ml, blunting of tubercles, slight irregulariti- ties in the inter-tubercular ridges (Fig. 3C) and edematous suckers were observed. This effect was progressively augmented using higher concentrations (60 & 80µg/ml) in the form of wrinkled surface, deeply fissured tegument with widespread loss of tubercles (Fig. 3D). Suckers were severely affected in the form of total disappearance of sucker vicinity (Fig. 3E) and tegumental erosion of oral sucker (Fig. 3F).

Juvenile *S. haematobium* stages: Tegument appeared corrugated with alternating bulging and depressions and invaginated acetabulum (Fig. 3G). Developed nodules and vesicles increased in size and depth, in proportion with dose and time, causing sloughing of the external tegument and accumulation of debris on and around worm body (Fig. 3H).

Discussion

Artemisinins and trioxolanes are among the firstly discovered anti-malarial drugs with promising anti-schistosomal effect (Le *et al,* 1982). Subsequently, more researchers tried to shed light on the anti-schistosomal powers of artemisinins and their synthetic derivatives (Keiser and Utzinger, 2007; Utzinger *et al,* 2007; Ho *et al,* 2014).Up on characterization of artemisinin chemically, a new class of anti-schistosomal bioactive peroxides named Ozonides (1,2,4-trioxolanes) started to exist. Their potency against *Schistosoma* species was discovered especially against the developing than the adult (Xiao *et al,* 2007).

The newly released anti-malarial combination of arterolane maleate (Ozonide OZ277) and piperaquine phosphate, SYN, exhibited encouraging anti-schistosomal properties against *S. mansoni* (Mossallam *et al,* 2015), which provides a good opportunity for curing *Schistosoma* and *Malaria* in co-endemic areas. In the same context, a proof-of-concept field trial had been done by Barda and his team to test the effect of multiple anti-malarial drugs including Synriam on *S. mansoni* and *S. haematobium* infected adolescents in Cote d' voir with comparable results (Barda *et al,* 2016).

To extend the available knowledge about this antimalarial drug, in vitro effect of ascending SYN concentrations on *S. haematobium* developmental stages were tested at selected time points. Since motility scoring, number of dead worms and tegument structural alterations are common parasitological parameters, which were often evaluated to indicate the biological activity of any anti-schistosomal drug (Sanderson *et al,* 2002; Pica-Mattoccia and Cioli, 2004; De Araújo *et al,* 2007; Boissier *et al,* 2009; Magalhães *et al,* 2009; Pinto-Almeida *et al,* 2016), these parameters were assessed to determine the effect of SYN on adult, early and late juvenile schistosomes in respect to the drug of choice, praziquantel.

In the present results, stages under study responded to SYN preparations according to "dose and time of exposure" pattern. This observation indicates that SYN can target all the intra-mammalian developmental stages of the parasite. This complies with similar studies on artemesinins tested for anti-schistosomal properties (Xiao *et al,* 2000; Portela *et al,* 2012; El-Beshbishi *et al,* 2013; 2015).

Up on comparing to the significant effect

of ascending concentrations of SYN, increasing the concentration of PZQ from 1µg/ml up to 30µg/ml didn't produce any discrepant anti-schistosomal effect on *S. japonicam* in vitro (Xiao *et al,* 2009). It was believed that ART-mediated lethal effect is due to alkylation of internal and external cellular proteins (enzymes), such as sarcoplasmic endoplasmic reticulum ATPase PfATP6, which is crucial to parasite survival (Eckstein-Ludwig *et al,* 2003). The carbon-centred free radicals incriminated in such process were produced by an irreversible redox reaction between the peroxides in artemisinins and hemoglobin degradation product, haem (Robert *et al,* 2002; Meshnick, 2002).

In the present study, SYN exhibited significant effect on juvenile stages of *S. haematobium.* The killing ability was detected at concentration of 10µg/ml after 1 hour of exposure, reaching the highest efficacy at 20µg/ml within 2 hours of incubation (100% mortality). However, PZQ had minimal effect on larval and immature stages. It was hypothesized that the juveniles' high potential gene activity in transcriptional up regulation of multidrug resistance-associated protein 1 (SmMRP1) can be responsible in such resistance against PZQ (Hines-Kay *et al,* 2012).

The present results were supported by other studies testing *in-vitro* anti-schistosomal properties of natural or synthetic ARTs on *S. mansoni*, *S. haematobium* and *S. japonicum* (Xiao *et al,* 1995; 2002; 2007). Eventually, early (skin stage) and late (hepatic) schistosomula were susceptible to SYN as evident by the process of pathological alterations in vivo. Mossallam *et al.* (2015) had proved that adding *S. haematobium* in culture media was more efficient than using SYN alone. The present results exhibited, beyond doubt, a slower onset of action of SYN on adult schistosomes using the lowest tested concentrations, while the effect was significantly augmented and hastened after the exposure to the higher doses (60 g/ml, 80 g/ml) without the addition of haem. This can be explained that haem was needed to intensify the effect of lower concentrations of SYN, while higher concentrations can produce enough killing effect by its one. However, their results using 20µg/ml SYN were apparently discrepant than ours using the same concentration within the same time point. This finding needs more trials to explain the pharmacokinetics and metabolism of the drug on the cellular level and its impact inside different strains of the parasite.

To document the effect of any anti-schistosomal drug, observations should be done on the ultrastructural level. Tegument, being the target for most of the anti-schistosomal drugs, was usually assessed using the electron micrograph studies. We observed prominent tegumental affection of juvenile and adult stages, which was escalated as the time of incubation and concentration of SYN increased. Bleb formation, vesiculation, focal erosions and disintegration were typical features observed in this study. Likewise, ARTs derivates' effect on the schistosomal tegument was reported clearly (Xiao *et al,* 2001; 2002; 2007; Utzinger *et al,* 2007). Besides, the tegumental damage reported with juvenile schistosomes was correlated with the high killing efficacy of SYN on larval stages.

Conclusion

SYN proved to a promising anti-schistosomal drug covering the inherited defects of PZQ in terms of effectiveness against different developmental stages of schistosomes, which provide both prophylaxis and treatment advantages. However, further studies are important to clarify the pharmacokinetics and bioavailability of the drug and consequently, to allocate suitable dose schedule in treating human infection.

Authors' contribution: All manuscript authors contributed to every activity of it; idea of paper, study design, collection of materials, methodology, writing the paper and revising it.

Conflict of Interest: The authors declared

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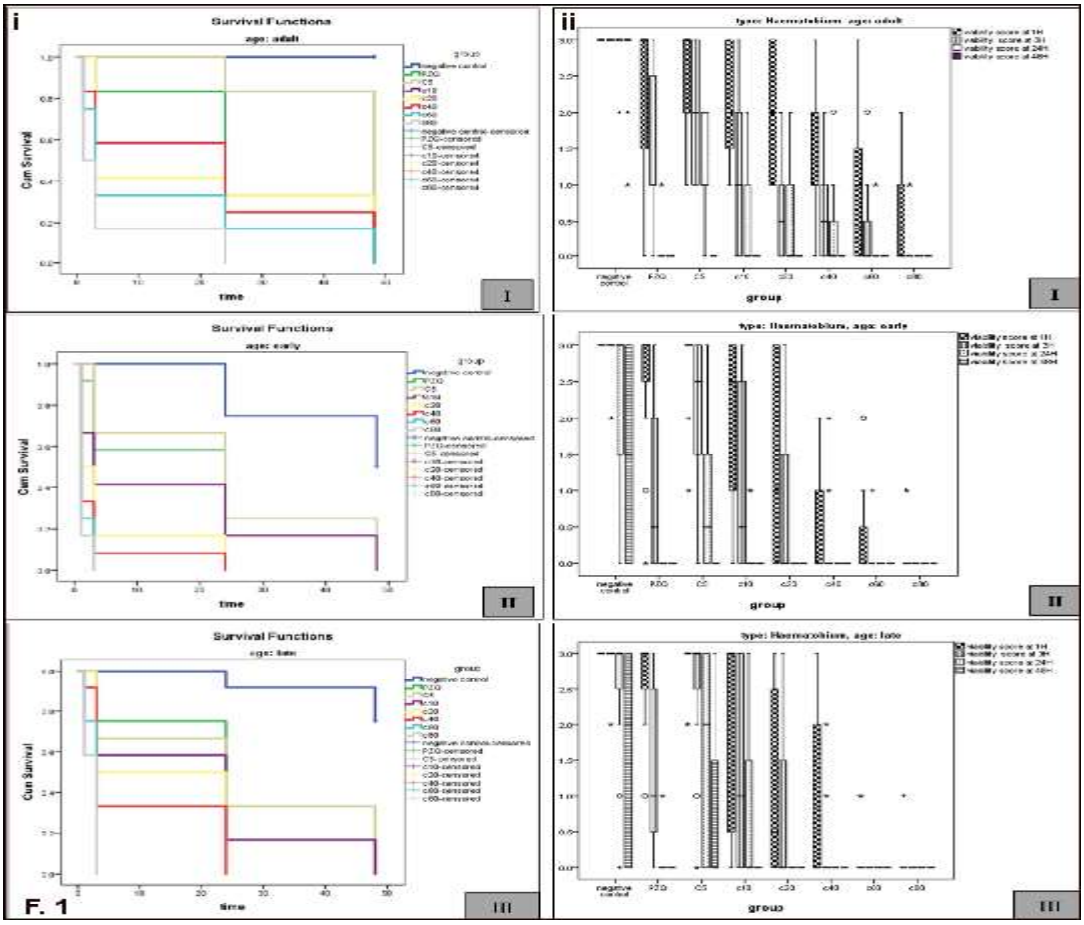
Explanation of Figures

Fig. 1: Effect of -time monitored- incubation with different concentrations (c5, c10, c20, c40, c60, & c80µg/ml) of SYN on study groups; (I) Adult *S. haematobium*. (II) Early schistosomula *S. haematobium*. (III) Late schistosomula or pre-adult *S. haematobium.* Panel (i): Kaplan-Meier survival analysis. Panel (ii): Viability score box-plot.

Fig. 2: SEM of *S. haematobium* developmental stages in control group. *Adult:* [A] Male and female in coplula showing normal tegumental (Tg) and body structure. [Ai](Insert) normal tubercles (Tb) size and arrangement with prominent healthy spines (Sp). [B] Normal adult oral (OS) and ventral suckers (VS). [C] Pre-adult Schistosomulae of control group with intact oral (OS) and ventral sucker (VS) and developing gynaecophoric canal (Gc).

Fig. 3: SEM of *S. haematobium* developmental stages after incubation with ascending concentrations of SYN. *Adult*: [A] Normal appearance of both oral (OS) and ventral suckers (VS). [B] Slight puffiness (Pf) of oral sucker and scattered vesicles (Vs) on the body tegument. [C] Blunting of tubercles in some areas, lost spines and irregularities (Ir) in inter-tubercular ridges. [D] Tegument showed wrinkled (Wr) surface, deeply fissured (Fr) tegument with widespread loss of tubercles. [E] Obliteration (Ob) of oral sucker concavity [F] Erosion (Er) of tegument covering oral sucker. *Immature:* [G] Disintegrated areas of tegument (Di) and Swollen sucker (Sw). [H] Sloughing of external tegument.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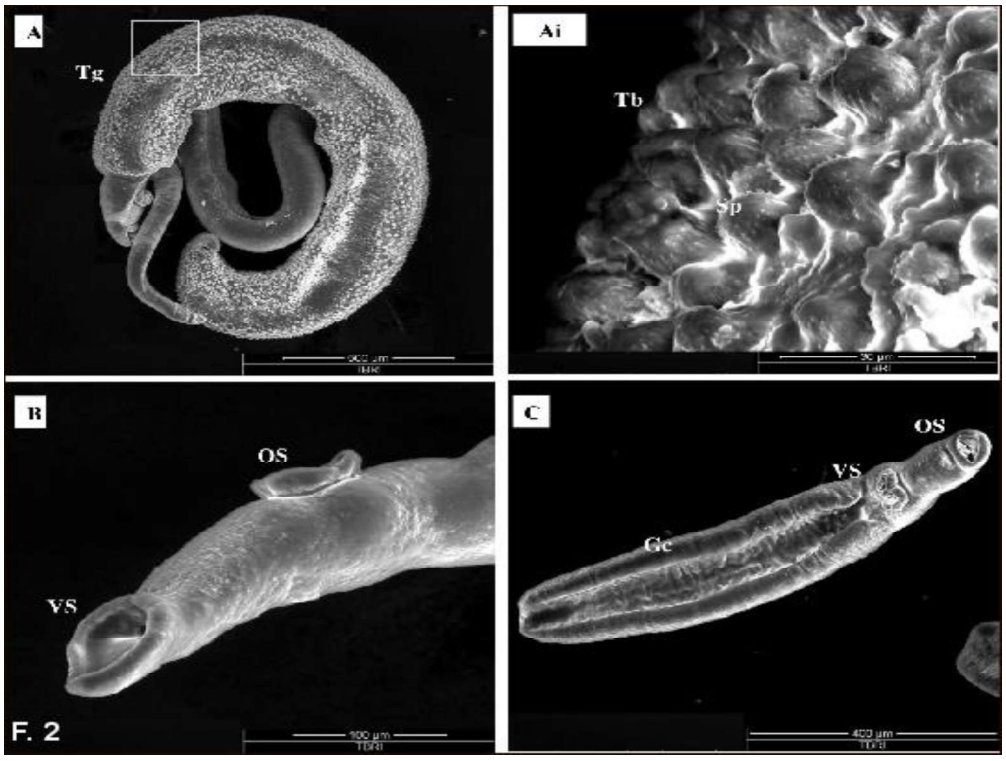
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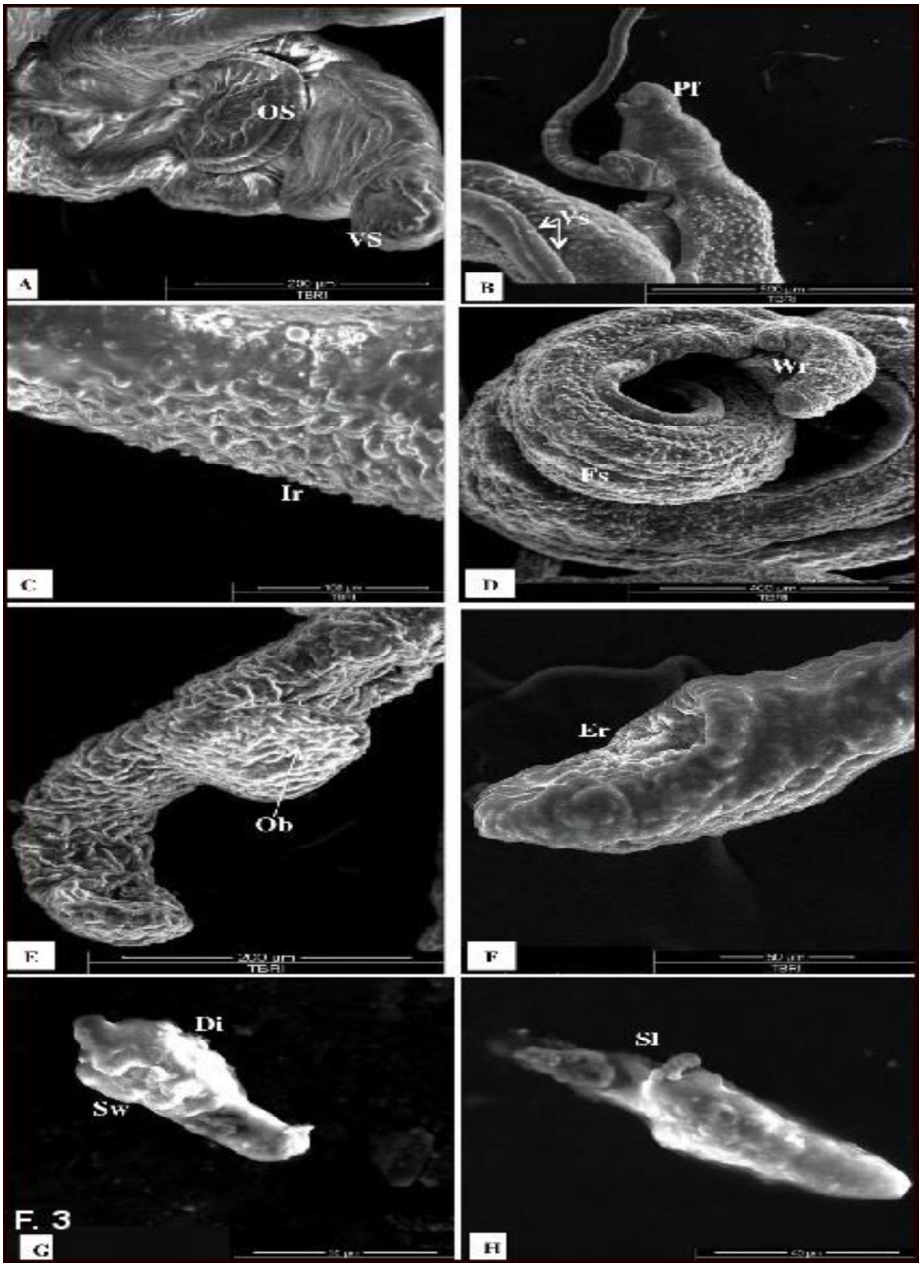
OS
VS
Gc

F. 2



F. 3