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Assessment of Testicular Histomorphometric Parameters and Reticular Fibres Density on Testicular Tissue of Diabetic Wistar Rat Placed on *Auricularia Polytricha*

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ABSTRACT

Role of *Auricularia polytricha* on diabetes – induced male reproductive dysfunction is not well understood. Local Nigeria men use this macro fungi to manage sexual dysfunction without an appropriate scientific investigations. The objectives of this study was therefore to investigate the effect of *A. polytricha* on histomorphometric parameters and reticular fibre density in testes of diabetic rat. Thirty (30) adult male Wistar rats was grouped into six designated A to F with 5 rats in each group. Group A (Normal Control) were treated with normal saline, Group B (administered with 65mg/kg.bw of streptozotocin) served as diabetic control. Groups C, D, E and F where placed on 250mg/kg.bw, 500mg/kg.bw, 1000mg/kg.bw of *A. polytricha* and 68mg/kg.bw of streptozotocin (STZ) respectively after inducing diabetics. Histomorphometric Measurements (seminiferous tubular diameter and germinal epithelial height) were done using ocular micrometre. Reticulum stain kits were used to demonstrate for reticular fibre. Results revealed that diabetic control group had remarkable increase in density of reticular fibre when compared to normal control and had tubular diameter (167.22 $\pm$ 0.10µm) that were significantly lower ($p<0.05$) when compared with normal control (310.81 $\pm$ 0.45 µm). Germinal epithelial height in Group B was also significantly ($p<0.05$) lower when compared to normal control. Reticular fibre expression was decreased in Groups C, D, E, and F in a dose dependent manner. However, tubular diameter and epithelial height were significantly higher ($p<0.05$) in groups placed on graded doses of *A. polytricha* when compared to diabetic control. Thickening of testicular interstitium as a result of reticular fibres density, and decrease in tubular diameter and epithelial height induced by diabetes were regulated and reversed following administration of graded doses of *A. polytricha.* Reversal in the reticular fibres expression may be due to powerful antioxidant potentials of *A. polytricha.* This study illustrates that supplementation with *A. polytricha* may confer protection for diabetes-related infertility.

Keywords: *Auricularia polytricha,* Diabetes, Histomorphometric parameters, Reticular fibers, seminiferous tubules, testes

1. Introduction

Diabetes will affect reproductive outcome of a fraction of diabetic men before and during their child-bearing ages (Saleh et al 2002). According to reports, approximately 50% of diabetic men will suffer from reproductive dysfunction within five years of the diagnosis (Saumya et al 2016). Consequently, it has become imperative to provide both preventive and curative measures to infertility in diabetic men. Furthermore, diabetes-related infertility has been ravaging a great population of young people with its attendant complications especially in rural communities where regular or periodic medical check is absent due to poverty and ignorance (Saleh et al., 2002).

Diabetes is thought to affect male reproductive function at multiple levels due to its effect on the endocrine control of spermatogenesis as well as penile erection and sperm quality. Studies in diabetic animal model have demonstrated an impairment of sperm quality and fecundity, structural defect and significantly lower motility of sperm cells (Baccetti et al., 2002), lower ability to penetrate zona free hamster eggs, detrimental effects on endocrine control of spermatogenesis and impairment of erection and ejaculation and progressive genotoxicity (Baker et al., 2004).

The gradual shift to herbal therapy with its attendant increasing acceptance, even among the elites confirm the claim that herbal remedies can provide cure for several diseases, including infertility in men (Killian et al., 2007). Many plant and animal products have been tested for possible fertility regulatory properties (Anthony, 2006). However, local Nigerian men have been reported to use *Auricularia polytricha* (wood ear mushroom) growing in farm lands and dead woods to manage sexual dysfunction with little or no research to confirm its efficacy

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the seminiferous tubules threaten the spermatogenesis and may lead to infertility (Bhatia et al., 2010)

A correlation between histo-morphometric parameters and reticular fibre expression can therefore be useful in assessing histopathological alterations in testes (Mallidis et al., 2011).

In this study, we evaluate the ameliorative effects of *A. polytricha* on histo-morphometric parameters and reticular fibre expression on testicular tissue of streptozotocin (STZ)-Induced diabetic rat model treated with graded doses of *A. Polytricha.*

2. Materials and Methods

2.1. Preparation of Extract

Auricularia Polytricha was obtained from Etomi central market located in Etung Local Government Area of Cross River State and authenticated at the Department of Biological sciences, University of Nigeria. The mushroom was dried at room temperature, powdered and subjected to crude extraction using the ethanol modified method. 200g of *A. polytricha* was soaked in 1000ml of ethanol, labelled, covered and allowed to sit for 72 hours, after which a clean filter paper (Watman No 1) was used to filter extracts. The filtrate was evaporated to dryness at 40^oC in a vacuum using a rotatory evaporator. The extract was weighed and stored at 4^0C.

2.2. Experimental Animals

This study carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Thirty (30) adult male Wistar rats with average weight of 150g were used for this research. The rats were kept in clean cages and divided into six groups designated A, B, C, D, E, and F with five rats in each group. The rats were allowed to acclimatize for two weeks in animal house, Department of Anatomy, Faculty of Medicine, University of Nigeria, Enugu Campus and allowed unrestricted access to commercially available chow (livestock feed) and water.

2.3. Experimental Design

Table 1. Experimental animals was divided into six (6) groups as follows:

GROUP	DESIGNATION	NUMBER OF ANIMALS	TREATMENT	DOSE
A	Normal control	5	Distilled water	3 mls
B	Diabetic control	5	Streptozotocin (STZ)	65mg/kg.bw
C	STZ + AP (Low Dose)	5	STZ + *A. polytricha*	250mg/kg.bw
D	STZ + AP (Mid Dose)	5	STZ + *A. polytricha*	500mg/kg.bw
E	STZ + AP (High Dose)	5	STZ + *A. polytricha*	1000mg/kg.bw
F	STZ + Metformin	5	STZ + Metformin	40mg/kg.bw

2.4. Induction of Hyperglycaemia

After fasting for twelve hours, diabetes was induced by administering streptozotocin (STZ) intra-peritoneally, reconstituted in 0.5M Sodium citrate and administered at a dose of 65mg/kg.bw (Ugochukwu and Babady 2003).

2.5. Confirmation of Diabetes

Diabetes was confirmed three days after administration of STZ using Accu-Check glucometer (Roche diagnostic, Germany) with blood samples obtained from tails of Wistar rats. Blood glucose levels (mmol/l) was checked before and after induction to ascertain hyperglycaemic state.

2.6. Administration of Extract

Experimental animals were kept for two weeks after inducing diabetes to allow for sustained hyperglycaemia after which *A. polytricha* extract administration commenced by oral gastric intubation and lasted for 21 days. The experimental protocol was maintained for a total of 35 days.

2.7. Termination of Experiment and Collection of Samples for Analysis

At termination, the animals were sacrificed with the testes removed and blotted with filter paper. Both right and left testes were weighed together and then suspended in Bouins fluid for fixation, preparatory to histological processing.

2.8. Histomorphometric Measurement

Histomorphometric Measurements were carried out using ocular micrometre after calibration on a light microscope. The stage micrometer was 1 mm long with 100 divisions so each division of the stage micrometer was one one-hundredth of a mm (0.01mm or 10 um). The eyepiece micrometer was divided into 100 units. 30 divisions of the reticle (eyepiece micrometer) corresponded to 200 micrometers. Measurements of seminiferous diameter and germinal epithelial heights were taken and recorded.

2.9. Reticular fibre examination

Reticulum stain kits - Modified Gomori's ((Sigma-Aldrich Products, Germany) was used in this research as a staining protocol for reticular fibre and basement membrane examination.

Silver nitrate solution was added in small amounts to the methenamine solution, mixing after each addition. A white precipitate formed which re-dissolved on shaking. Stock solution used was clear. The stock solution was filtered into a brown bottle and stored at 4^oC.

Procedure:

Slides were deparaffinised and then put into distilled water and then oxidize in 0.5% periodic acid solution for 15 minutes at room temperature. Slides were rinsed again 3 times in distilled water then incubated in methenamine silver working solution for 1 hour at 60^oC. Slides brearing tissue sections were then rinsed in hot distilled water followed by water at room temperature. Sectioned were toned in gold chloride and rinsed again in distilled water, treated with sodium thiosulfate for 2 minutes, rinsed and counterstained in nuclear fast red. Sections were finally dehydrated in xylene and mounted on a microscope.

Results showed black for basement membrane and reticular fibres with pink or green nuclear background.

2.10. Statistical Analysis

Quantitative data from this research was recoded and tabularised. Statistical significance of the differences between the groups was determined using one-way analysis of variance (ANOVA) done with SPSS, version 20 statistical analysis program. P<0.05 was considered as significant.

3. Results

3.1. Reticular fibre and basement membrane examination

Methenamine Silver (Gomori PAM) staining protocol for reticular fibre and basement membrane examination on testicular tissue was negative in group A (Normal Control) as there was no reticular fibre expressed in the basement membrane (Figure 1). However, observation showed positive result in group B (Diabetic control) which revealed the black colour reticular fibre seen round the seminiferous tubules (Figure 2). Density of reticular fibre was also increased in the diabetic control animals. Reticular fibre density in the section of testis in diabetic animals placed on 250mg/kgbw of and 500mg/kgbw of *A. polytricha* (Group C and D) was not remarkably decreased (Figure 3 and 4). Even though the result was positive, reticular fibre density was not as remarkable as that expressed in the diabetic control animals. Section of testis in groups E and F showed negative result with methenamine silver stain (Figure 5 and 6).

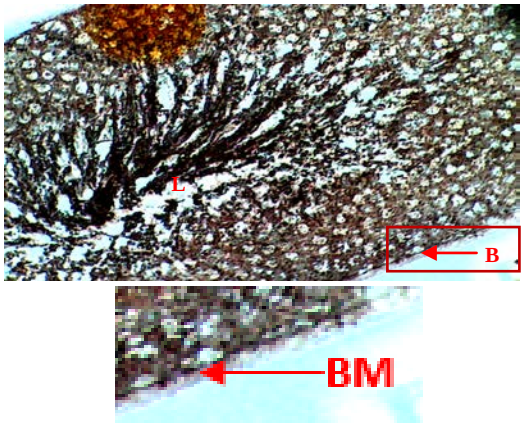

Figure 1. GROUP A (Normal Control) X400

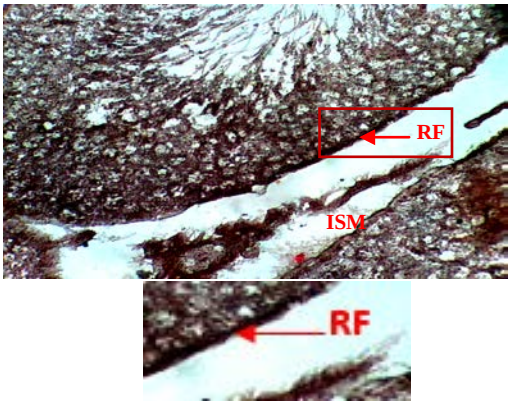

Figure 2.GROUP B (Diabetic Control) - X400

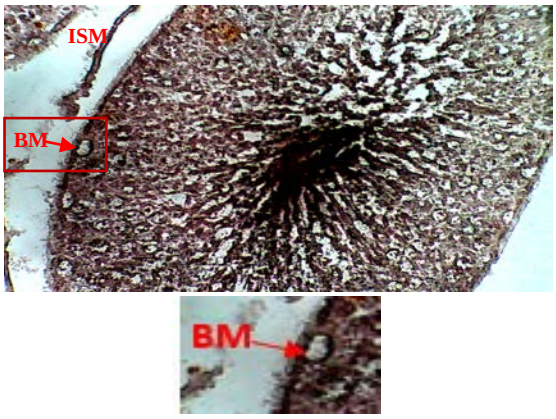

Figure 3. GROUP C - STZ + AP (250 mg/kg.bw) X400

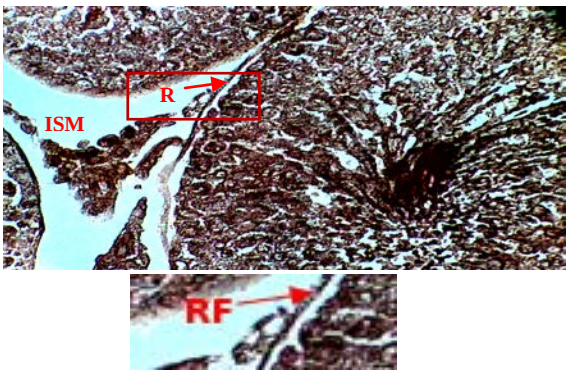

Figure 4. GROUP D, STZ + AP (500mg/kg.bw) X400

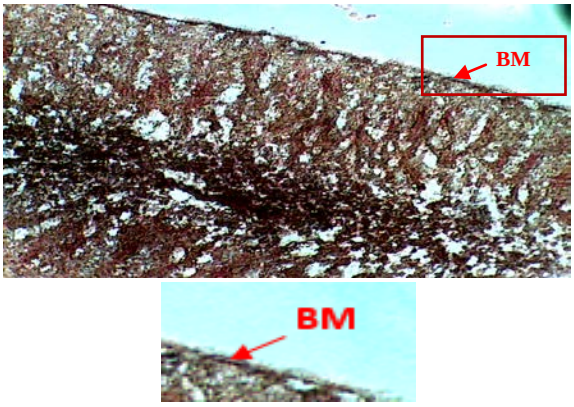

Figure 5. GROUP E - STZ + AP (1000mg/kg.bw) X400

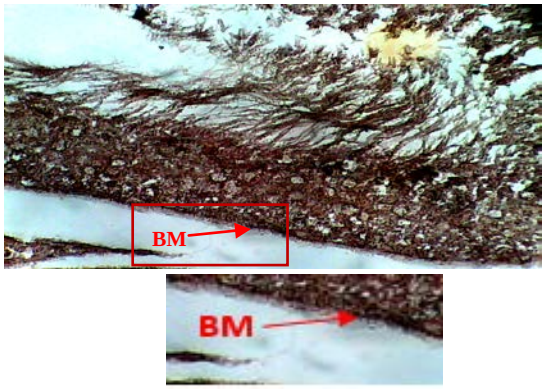

Figure 6 . GROUP F (STZ + Metformin) X400
Section of testis showing decreased formation of reticular fibres

KEY : L – Lumen , ISM – Testicular Interstitium , BM – Basement Membrane , RF – Recticular Fibres.

3.2. Histo-Morphometric Parameters

3.2.1. Tubular Diameter

Seminiferous tubular diameter and germinal epithelial height were the histomorphometric parameters considered in this study. 167.22 ± 0.10µm recorded as diameter of seminiferous tubules in Group B (Diabetic Control) was significantly decreased when compared to 310.81 ± 0.45µm in Group A (Normal Control). Section of testis in group C did not show any significant difference ($p<0.05$) in terms of tubular diameter when compared to Group B. However, among the diabetes induced groups, animals placed on 500 and 1000mg/kgbw of *A. polytricha* showed significantly (at $p<0.05$) increased tubular diameter (210.22 ± 0.45 and 249.10 ± 0.33 respectively) when compared to low dose group (Figure 7)

3.2.2. Germinal epithelial heights

Germinal epithelial heights were least in groups B (Diabetic control) and group C (Diabetic animals that received 250mg/kgbw of *A.* polytricha), measuring 10.11 ± 0.55 µm and 10.50 ± 0.72µm respectively, and were considered significantly decreased ($p<0.05$) when compared to epithelial height in Normal control - Group A (19.03 ± 0.23µm). Group E (1000mg/kgbw of *A.* polytricha) recorded the highest value in epithelial height (15.00 ± 0.34µm) among the diabetic groups and these values were significantly ($p<0.05$) higher when compared to Diabetic control - Groups B and C. However, interventions in Group D (500mg/kgbw of *A.* polytricha) and F (standard antidiabetic drug – metformin) showed a significant increase in the height of germinal epithelium when compared to the diabetic control (Figure 8).

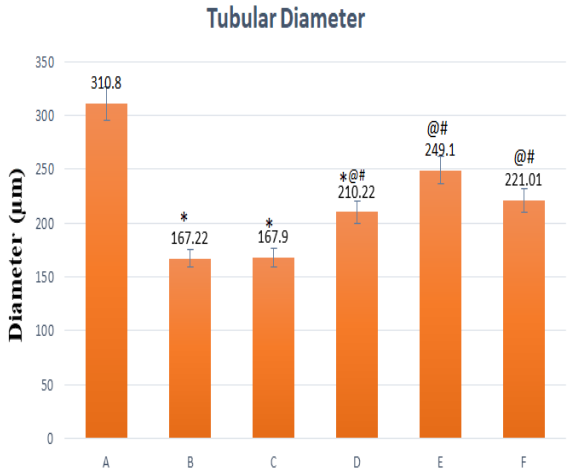

Figure 7: Comparison of Tubular Diameter in the different experimental groups.

Values are expressed in Mean ± SEM. N = 5.

***** = Values are significantly decreased when compared to Normal Control at $p<0.05$. **@** = Values are significantly increased when compared to Diabetic Control at $p<0.05$.# = Values are significantly increased when compared to Low Dose group at $p<0.01$.

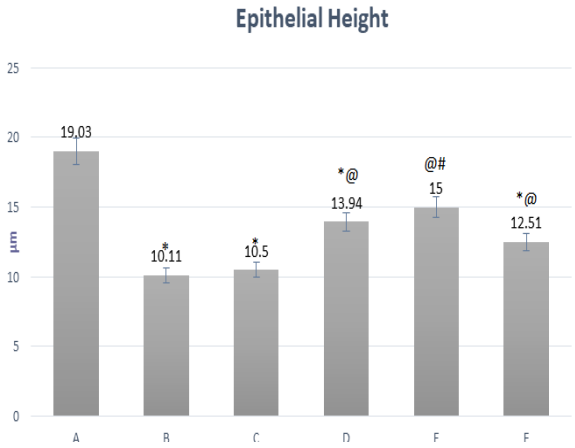

Figure 8: Comparison of Germinal Epithelial Height in the different experimental groups.

Values are expressed in Mean ± SEM. N = 5.

***** = Values are significantly decreased when compared to Normal Control at $p<0.05$. **@** = Values are significantly increased when compared to Diabetic Control at $p<0.05$.# = Values are significantly increased when compared to Low Dose group at $p<0.01$.

4. DISCUSSION

The effect of *A. polytricha* on testicular histomorphometric parameters and reticular fibre density in diabetic rat was investigated in this study. It was observed that seminiferous diameter and germinal epithelial heights which were significantly lower in diabetic control rats when compared to normal control group increased significantly following treatment with *A. polytricha*. Thickening of reticular fibre in diabetic animals was reversed following *A. polytricha* administration. From the aforementioned result, it is evident that diabetes have caused increase in the density of reticular fibre in the basement membranes of seminiferous tubules. The underlying mechanism may be connected with generation of reactive oxygen specie (ROS) in sustained hyperglycaemic condition capable of initiating a cascade of degenerative process that may result in altered structure of basement membrane. Another possible mechanism could be associated with the effect of hyperglycaemia on somatic cells due to rapid destruction of cell membranes via lipid peroxidation. Andreyev *et al.*, (2005) reported that excessive generation of ROS in hyperglycaemic condition, without a corresponding balance in antioxidant production capacity may cause cellular damage.

Correlation between extracellular matrix expansion as a result of increased expression in reticular fibres density and some histomorphometric parameters especially seminiferous tubular diameter and germinal epithelial height are considered significant variables in assessing testicular tissue damage and possible reversal. Increased reticular fibres density around the seminiferous in testicular tissues of diabetic control group as observed in this study indicates matrix expansion and as a consequence, smaller diameter of the seminiferous tubules and decreased germinal epithelial heights. This may imply that the higher the reticular fibres density, the wider the interstitial matrix and the lower the integrity of basement

membrane. Spermatogonia located at the basal compartment differentiates while moving to the adluminal compartment and a stable basement membrane is required for this cellular migration (Kilian et al., 2007). Increase in reticular fibre around the seminiferous tubules threatens the integrity of basement membrane and may affect spermatogenic process leading to infertility (Kilian et al., 2007; Baker et al 2004)

Decrease in density of reticular fibre and higher values of tubular diameter and epithelial heights following observed in diabetic animals following administration of *A. polytricha* may have been due to a great number of therapeutic, nutritive and biologically active components in *A. polytricha* that may have given protection against tissue degeneration.

Reactive oxygen species (ROS) which are increased by hyperglycaemia both in type 1 and type 2 diabetes, will cause oxidative stress (Beckman et al.,2001), which in turn plays a role in the development of complications in diabetes (Baynes et al., 1999). Chang et al., (2011) and (Mau et al., 2001) reported that *A. polytricha* is capable of inhibiting lipid peroxidation in body organs thereby restoring their weight and are known exogenous source of antioxidant because of the presence of bioactive β-D-glucan polysaccharides which are the major therapeutic and nutritional components found in cell walls of *A. polytricha.*

Findings from Chen et al., (2011) and Sun et al., (2010) reveal that uronic acid has been fractionized from fruiting body of *A. polytricha* and that the higher the uronic acid content of β-D-glucan polysaccharides, the more effective the antioxidant activity of the polysaccharide. Liao et al., (2014) and Chen et al., (2008) demonstrated that *A. polytricha* polysaccharides improved significantly, total antioxidant capacity and lipoprotein lipase activity in mice but was found to reduce melondialdehyde levels and arteriosclerosis index in rats and he attributed the strong antioxidant status to the phenolic compound in *A. polytricha*.

Photomicrographs of sections of testicular tissue in groups D, E and F placed on 500mg/kg.bw, 1000mg/kg.bw *A. polytricha* and STZ respectively revealed a reversal in reticular fibres expression and recorded significantly ($p<0.05$) higher tubular diameter and germinal epithelial heights when compared to the diabetic control. Some underlying mechanisms may have been responsible. Firstly, *A.* polytricha is a powerful exogenous source of antioxidants. Luo et al., 2009 reported that polysaccharides fractionated from *A. polythrica* formulation proof to have potential antioxidant effect against hydroxyl and superoxide radicals. This may have been responsible for inhibiting lipid peroxidation and improve on the microstructure of testicular tissues. Chen et al., 2011 reported similar findings. Secondly, the protein content in *Auricularia species* of mushroom was found to have significant amount of glutamic acid, lysine, alanine, glutamic acid, serine, and threonine and other essential free amino acids which may have provided the needed nutritional and therapeutic ingredients needed for repairs.

5. Conclusion

Findings from this study suggest that ethanolic extract of *A. polytricha* can reverse altered histomorphometric parameters and reticular fibre density in testicular tissue of diabetic rat model. This reversal was dose dependent. Clinical application of this research may suggest supplementation with *A. polytricha* on individuals with diabetes related infertility.

5. Ethical clearance

Ethical clearance was obtained from the Ethical Committee, Faculty of Basic Medical Sciences, University of Nigeria, Enugu Campus, Nigeria

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Competing Interest

All authors in this research are not by any means linked to any funding body and so there was a complete absence of external influence.

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