Original Article

Stereological Quantification of Leydig and Sertoli Cells: Technique of Assessing Antifertility Potentials of Metronidazole

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ABSTRACT
Changes in the numbers or volume of the different cell types in the testis have been widely used to ascertain the effects of environmental and chemical agents on the testis. This study is designed to investigate the direct effects of metronidazole on the testicular cells by quantifying the number of Sertoli and Leydig cells. A total of 105 adult male and 50 female wistar rats weighing 170±10g (70-90 day old) were used for the experiment. The rats were randomly divided into a control and experimental groups. There were four major groups with 5 subgroups consisting of 5 rats each. Varying doses of metronidazole were used depending on the experiment. Experiment 1; animals were fed with 15mg/kg of metronidazole, experiment 2; fed with 30mg/kg of metronidazole, experiment 3; administered with 200mg/kg of metronidazole and experiment 4; fed with 400mg/kg of metronidazole. Each experimental has 5 sub-groups; A control, B; group fed with the experimental dose, C; experimental dose with vitamin E, D; experimental dose with testosterone, E; fed with experimental dose, vitamin E and testosterone and sub-group F, a reversal group which was left for 8weeks after cessation of treatment. At the end of the experiment, animals were scarified and the antioxidant effects of metronidazole were investigated using malonildialdehyde concentration and catalase activity. Results showed that metronidazole at the therapeutic dose of 15mg/kg and 30mg/kg did not have significant negative effect on the testicular cell number. At the dose of 200mg/kg and 400mg/kg, there was a marked increase in number of Sertoli and Leydig cells. The results of this study indicate that metronidazole administration (200mg/kg or 400 mg/kg), for 8weeks, caused a reduction in the total number of Sertoli and Leydig cells of the testis and this effect was reversible after 8weeks of abstinence.
KEY WORDS: stereology, Leydig cells, Sertoli cells

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INTRODUCTION

Stereology is an interdisciplinary field that is largely concerned with the three dimensions of planar sections of materials or tissues. It provides a practical techniques for extracting quantitative information about a three dimensional material from measurements made on two dimensional planar sections of the material. It is a method that utilizes random, systemic sampling to provide unbiased and quantitative data. It is an important and efficient tool in many applications of microscopy in areas of histology, neuroanatomy, histopathology, haematology etc**.**

During the last few decades the effects of different environmental and chemical agents on the testis have been investigated in animal experiments. Changes in the numbers or volume of the different cell types in the testis have been widely used end points in these studies. However, careful analyses of the methods used in quantitative studies are needed before the conclusions of these investigations can be accepted.[1] Generally speaking, quantification in the testis has been carried out by profile counting in semi thin sections, measurement of relative areas, or ratios of the cell type studied. Most of the methods used are known to result in biased estimates (e.g., because the number of profiles counted in the sections depend on the size and shape of the structure counted .[2] Based on assumptions about the shape and size of the structures, various mathematical correction formulas have been applied to correct for these biases in conventional profile counting designs.[2,3]

During the past few decades new stereological tools have been introduced. One of the new methods, optical fractionation, which is based on a systematic uniform sampling scheme, a fractionator design, and a 3-dimensional counting probe, the optical dissector, has proved to be a highly efficient stereological method with which to estimate the total number of cells in an organ, such as the testis. [4,5]

Infertility is a major reproductive health problem in many countries in Sub-Saharan Africa, Nigeria included.[6] and it is the inability of a couple (male/female unit) to achieve a pregnancy after one year of regular unprotected sexual exposure.[7]

In Nigeria, infertility is the commonest reason for gynaecological consultation and most gynaecologists assess that considerable proportion of their time is spent attending to individuals/couples who have primary or secondary infertility.[6] Studies in sub-Saharan Africa indicate that both primary and secondary infertility are commoner here than in Europe and United States of America.[8] Whereas high fertility rates are found in women in the Sub-Saharan African zone, higher infertility rates are also seen here compared to Caucasian countries.

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Nigeria, with a population of about 150 million people, has a high population growth rate and also a high rate of fertility, six births per woman in 2005.[9] Available evidence also suggests that the country has high rates of primary and secondary infertility. Community based data suggest that up to 30 per cent of couples in some parts of Nigeria may have proven difficulties in achieving a desired conception after two years of marriage without the use of contraceptives.[10]

By contrast to the high prevalence of male and female causes of infertility in Nigeria, there are currently limited treatment options available for infertile couples. The conventional treatment of infertility in tertiary institutions in Nigeria often does not have a success rate better than ten per cent. [8] and there are currently very few private centres for the specialized treatment of the more difficult cases of infertility in this region. The services of these centres are beyond the reach of the ordinary citizens. More specifically, whereas there are now programs that seek to reduce the high rate of fertility in Nigeria, there are at present increasing number of causes of infertility. This study therefore, is design to ascertain the infertility effects of metronidazole by stereologically quantifying the number of Sertoli and Leydig cells in the testis of the rat.

MATERILAS AND METHODS

Study site

The experimental male wistar rats were bought and housed in the animal house located in the College of Health Sciences, Niger Delta University, Wilberforce Island. This study site was chosen because similar studies had been carried out successfully in this centre such as the antifertility effects of *Abrus Precatorius* on the testes of male rats.

Metronidazole, vitamin E and Testosterone were purchased from Cynflac Pharmacy, hospital road, Yenagoa.

ANIMALS AND TREATMENT

A total of 105 adult male and 50 female wistar rats weighing 170±10g (70-90 day old) were obtained from the Animal house of the College of Health Sciences, Niger Delta University, Wilberforce Island. There were maintained in 12 h light and 12h dark conditions at a temperature of 27ºC-30ºC in the animal house. The standard laboratory chew and tap water were available *ad libitium*. After the acclimatization period of two weeks, the rats were randomly divided into a control and experimental groups. There were four major groups with 5 subgroups consisting of 5 rats each.

Experiment 1: using adult rats fed with 15mg/kg of metronidazole

In experiment 1, adult male rats weighing 175g±5, about 70-90 days old at the commencement of the experiment were used. There were divided into a control group (Group1A), a group treated with 15mg/kg of metronidazole (1B), a group fed with 15mg/kg of metronidazole and 400mg/kg/day of vitamin E concurrently for 8weeks (Group 1C), a group treated with 15mg/kg of metronidazole and 0.36mg/kg/day of testosterone (Group 1D), another group treated with 15mg/kg of metronidazole, 400mk/kg/day of vitamin E and 0.36mg/kg/day of testosterone concurrently (Group 1E). A reversal group (Group 1F) was left for 8weeks after cessation of treatment with metronidazole to see whether the observed effects were reversible. Each group had 5 rats randomly divided into the groups. The metronidazole was delivered as a single dose in 0.1ml of distrilled water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for fertility after the treatment.

Experiment 2: using adult rats fed with 30mg/kg of metronidazole

In experiment 2, adult male and female rats weighing 160g±0.5, about 70-90 days old at the commencement of the experiment were used. There were divided into a control group (Group2A), a group treated with 30mg/kg of metronidazole (2B), a group treated with 30mg/kg of metronidazole and 400mg/kg/day of vitamin E concurrently for 8weeks (Group 2C), a group treated with 30mg/kg of metronidazole and 0.36mg/kg/day of testosterone (Group 2D), another group treated with 30mg/kg of metronidazole, 400mk/kg/day of vitamin E and 0.36mg/kg/day of testosterone concurrently (Group 1E). A reversal group (Group 1F) was left for 8weeks after cessation of treatment with metronidazole to see whether the observed effects were reversible. Each group had 5 rats randomly divided into the groups. The metronidazole was delivered as a single dose in 0.2ml of distrilled water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for fertility after the treatment.

Experiment 3: using adult rats fed with 200mg/kg of metronidazole

In experiment 3, adult male rats weighing 170g±0.9, about 70-90 day old at the commencement of the experiment were used. The rats five in each group were randomly divided into a control group (Group3A), a group treated with 200mg/kg of metronidazole (3B), a group fed with 200mg of metronidazole and 400mg/kg/day of vitamin E concurrently for 8weeks (Group 3C), a group treated with 200mg/kg of metronidazole and 0.36mg/kg/day of testosterone (Group 3D), another group treated with 200mg/kg of metronidazole, 400mk/kg/day of vitamin E and 0.36mg/kg/day of testosterone concurrently (Group 3E). A reversal group (Group 3F) was left for 8weeks after cessation of treatment with metronidazole to see whether the observed effects were reversible. The metronidazole was delivered as a single dose in 0.625ml

of distilled water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for fertility after the treatment.

Experiment 4: using adult rats fed with 400mg/kg of metronidazole.

In experiment 4, adult male rats weighing 200g±5, about 70-90 week old at the commencement of the experiment were used. There were divided into a control group (Group4A), a group treated with 400mg/kg of metronidazole (4B), a group treated with 400mg/kg of metronidazole and 400mg/kg/day of vitamin E concurrently for 8weeks (Group 4C), a group treated with 400mg/kg of metronidazole and 0.36mg/kg/day of testosterone (Group 4D), another group treated with 400mg/kg of metronidazole, 400mk/kg/day of vitamin E and 0.36mg/kg/day of testosterone concurrently (Group 4E). A reversal group (Group 4F) was left for 8weeks after cessation of treatment with metronidazole to see whether the observed effects were reversible. Each group had 7 rats randomly divided into the groups. The metronidazole was delivered as a single dose in 0.625ml of distrilled water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for fertility after the treatment.

Dose of Metronidazole

The dose of 400mg/kg and 200mg/kg respectively was selected because the LD50 of metronidazole (p.o) was determined and it was found to be 5000mg/kg.[11] The 400mgkg dose taken in this study is less than 1/8 of the lethal dose and 200mg/kg is less than 1/16 of the lethal dose. Besides, from literature, several other authors had used similar dose.[12,13]

Route of administration

The tablet form of metronidazole and vitamin E were administered through the naso-gastric rout while the testosterone injection was given intramuscularly.

Retrieval of tissues

At termination, the rats were anaesthetized with ketamine 1 mg/kg [intramuscularly (i.m.)], testicular tissues were collected in buffered bouin solution for histology.

STEREOLOGICAL METHOD

Estimation of total Sertoli and Leydig cell number in intact rat testes

This was determined according to the method earlier described by *Petersen et al (1996).*[14]

1. The entire testis was fixed in a fixative containing 20 ml formaldehyde 40% and 4 ml acetic acid and 76 ml water. From each testis, a known fraction of the tissue was sampled systematically with a random start in a careful stepwise sampling procedure: (a) Each testis was cut into 4-mm-thick slabs, providing 8-12 slabs; (b) Every 2nd-3rd slab was sampled systematically randomly and cut into 4-mm-thick bars providing 6-10 bars; (c) every 2nd-3rd bar was sampled and cut into cubes; (d) every 4th to 6th of these cubes (approximately (approximately 8-10 cubes) were sampled.
2. The sampled tissue was embedded in a paraffin wax and stained with hematoxylin and eosin where the Leydig cells and Sertoli cells are identified.
3. The blocks of paraffin was, each containing 8-10 cubes of testicular tissue, were cut into 40-µm thick sections with use of a microtome.
4. Approximately 10 sections were sampled from each testis and the optical fractionator principle used to estimate the total number of Sertoli and Leydig cells in a known fraction of the tissue. To avoid bias bias from cutting artefacts a disector height of 15µm was chosen. By this sampling procedure the coefficient of error (CE = SEM/mean) at each sampling level is estimated and kept below 10%.
5. Approximately 150 of each cell type in each testis was counted. The Sertoli cells were recognised in the seminifereous tubules by their pale invaginated irregular nuclei with a prominent nucleolus. The Leydig cells were recognised in the interstitium as relatively large ovoid shaped cell with an eccentric nucleus. The nucleus contained a prominent nucleolus and peripherally localised chromatin. To obtain approximately the same sample size different sampling frame sizes were used for Sertoli and Leydig cells. The frame size for Sertoli cells were approximately 600 µm2 and for Leydig cells 2700 µm2.
6. The total number of Sertoli and Leydig cells were estimated by multiplication of the counted number of cells (µQ) by the inverse of the sampled fraction. For example in one testis every 2nd slab, every 3rd bar, every 6th cube, and every 12th methacrylate section were sampled and counting of Leydig cells was performed in 1/367 of the sampled tissue section. Thus the global sampling fraction was 1: (2x3x6x12x367) and the total number of Leydig cells was the inverse sampling fraction multiplicated with µQ

STATISTICAL ANALYSIS

Data are expressed as mean ± SD and the test of significance analyzed by the student's *t*-test. The differences were considered significant at $p < 0.05$.

Ethical considerations

The research proposal was submitted to the Ethical Research Committee of the College of Health Sciences of the University of port-Harcourt for consideration and approval before commencement of this research work.

RESULTS

Total number of Sertoli and Leydig cells in control and metronidazole treated rats

The total numbers of Sertoli and Leydig cells are not markedly reduced in both 15mg/kg and 30mg/kg of metronidazole treated rats as shown in tables 1 and 2. However, there were a markedly reduced total number of Sertoli and Leydig cells in experimental groups 3 treated with 200mg/kg of metronidazole and experimental group 4 which was fed with 400mg/kg of metronidazole as shown in table 4 and 5. The effects were not pronounced in the sub-groups in which metronidazole was co-administered with vitamin E. The values in all the reversal groups were brought back to near normal or normal levels in comparison to the control.

Table 1. Morphometric parameters- number of Sertoli and Leydic cells in control and rats treated with 15mg/kg of metronidazole

GROUP/ DURATION	NUMBER OF SERTOLI CELLS COUNTED	TOTAL NUMBER OF LEYDIG CELLS X10 ^{6}	NUMBER OF LEYDIG CELLS COUNTED	TOTAL SERTOLI CELL NUMBER X10^{6}
1A	**95**	**151**	**34**	**53.9**
1B	**91**	**144.7**	**28**	**44.4**
1C	**84**	**133.6**	**21**	**33.3**
1D	**80**	**127.2**	**27**	**42.9**
1E	**93**	**147.9**	**26**	**41.3**
1F	**96**	**152.6**	**38**	**60.4**

1A; control, 1B; fed with 15mg/kg of metronidazole, 1C; fed with 15mg/kg metronidazole and 400mg/kg of vitamin E, 1D; fed with 15mg/kg of metronidazole and 0.36mg/kg of testosterone, 1E; fed with 15mg/kg of metronidazole, 400mg/kg of vitamin E and 0.36mg/kg of testosterone, 1F; reversal.

Table2. Number of Sertoli and Leydic cells in control and rats treated with 30mg/kg of metronidazole

GROUP/ DURATION	NUMBER OF SERTOLI CELLS COUNTED	TOTAL NUMBER OF LEYDIG CELLS X10 ^{6}	NUMBER OF LEYDIG CELLS COUNTED	TOTAL SERTOLI CELL NUMBER X10^{6}
2A	**95**	**151**	**34**	**53.9**
2B	**93**	**147.9**	**35**	**55.7**
2C	**87**	**138.3**	**29**	**46.1**
2D	**89**	**141.5**	**33**	**52.5**
2E	**103**	**163.8**	**36**	**57.2**
2F	**109**	**173.3**	**41**	**65.2**

2A; control, 2B; fed with 30mg/kg of metronidazole, 2C; fed with 30mg/kg metronidazole and 400mg/kg of vitamin E, 2D; fed with 30mg/kg of metronidazole and 0.36mg/kg of testosterone, 2E; fed with 30mg/kg of metronidazole, 400mg/kg of vitamin E and 0.36mg/kg of testosterone, 2F; reversal.

Table 3. Number of Sertoli and Leydic cells in control and rats treated with 200mg/kg of metronidazole

GROUP/ DURATION	NUMBER OF SERTOLI CELLS COUNTED	TOTAL NUMBER OF LEYDIG CELLS X10 ^{6}	NUMBER OF LEYDIG CELLS COUNTED	TOTAL SERTOLI CELL NUMBER X10^{6}
3A	**95**	**151**	**34**	**53.9**
3B	**81**	**128.8**	**29**	**46.1**
3C	**93**	**147.9**	**33**	**52.5**
3D	**103**	**163.8**	**31**	**49.3**
3E	**100**	**159**	**35**	**55.6**
3F	**94**	**149.5**	**33**	**52.2**

3A; control, 3B; fed with 200mg/kg of metronidazole, 3C; fed with 200mg/kg metronidazole and 400mg/kg of vitamin E, 3D; fed with 200mg/kg of metronidazole and 0.36mg/kg of testosterone, 3E; fed with 200mg/kg of metronidazole, 400mg/kg of vitamin E and 0.36mg/kg of testosterone, 3F; reversal.

Table 4. Number of Sertoli and Leydic cells in control and rats treated with 400mg/kg of metronidazole

GROUP/ DURATION	NUMBER OF SERTOLI CELLS COUNTED	TOTAL NUMBER OF LEYDIG CELLS X10 ^{6}	NUMBER OF LEYDIG CELLS COUNTED	TOTAL SERTOLI CELL NUMBER X10^{6}
4A	**95**	**151**	**34**	**53.9**
4B	**54**	**48**	**18**	**28.6**
4C	**63**	**56**	**21**	**33.4**
4D	**78**	**69**	**31**	**49.3**
4E	**86**	**76**	**32**	**50.9**
4F	**107**	**95**	**37**	**58.8**

4A; control, 4B; fed with 400mg/kg of metronidazole, 4C; fed with 400mg/kg metronidazole and 400mg/kg of vitamin E, 4D; fed with 200mg/kg of metronidazole and 0.36mg/kg of testosterone, 4E; fed with 200mg/kg of metronidazole, 400mg/kg of vitamin E and 0.36mg/kg of testosterone, 4F; reversal.

DISCUSSION

The number of Sertoli cell in the testes from adult males is approximately 151 x 10^6 and the number of Leydig cells approximately 59.3 x 10^6. The estimated total Sertoli cell numbers have been reported to range from 390 to 3700 x 10^6 and the total Leydig cell number from 400 to 800 x 10^6 [15,16] The total Leydig and Sertoli cell number estimated by the use of stereological method differs from results obtained from most previous studies. This large range may be ascribed to difficulties in interpretation of results from assumption based design [17] and secondly, in the sexually matured testis, two types of Sertoli cells can be identified at the electron microscope level.

Type A Sertoli cells have light staining nucleus, and contain numerous filaments and ribosomes, abundant smooth endoplasmic reticulum (SER), and typical junctions with adjacent Sertoli cells and germ cells.[18] These cells extend from the basement membrane to the lumen of the tubule and are routinely classified as Sertoli cells even at the light microscope level.

Type B Sertoli cells are less numerous and are found only in close apposition to the basement membrane of the seminiferous epithelium. They are small cells with dark nuclei and limited cytoplasm containing SER and numerous filaments. [18] At the light microscope level, type B Sertoli cells are difficult to identify as they are similar in appearance to adjacent differentiating spermatogonia.

Therefore, it is probable that studies evaluating Sertoli cells in the sexually mature rat at the light microscope level may evaluate only type A Sertoli cell nuclei. The functional significance of type B Sertoli cells is presently unknown. In addition, stereological evaluation of Sertoli cells using light microscopy is a labour-intensive process [19,20] , and therefore, a marker that specifically stains both types of Sertoli cell nuclei would have greatly facilitated the speed and accuracy of stereological enumeration procedure.

It has also been observed that the imprecision of the estimates of Sertoli and Leydig cells should be seen in view of the observation of apparently very large biological variations. The very large variation implies that even the relatively large impression of estimate will be acceptable in most clinical situation and that the most important factor in clinical studies comparing groups of patient will be the number of individual included.[21] In this study, total number of Leydig and Sertoli cells in the various experimental groups correlate with that of sperm analysis, fertility test, histology and hormonal profiles of metronidazole treated rats.[22]

CONCLUSION

We have described the estimation of total number as an example of the use of stereological methods. In this design using paraffin wax embedding other parameters such as mean cell volume, surface area of cells or length of structures can also be quantified and based on comparing values of present study with values of other parameter of previous studies, total number of testicular cells estimated stereologically may be used as a function of fertility potentials.

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