HISTOMETRIC INVESTIGATIONS ON THE TESTICULAR TISSUE OF RATS WITH ALLOXAN DIABETES AND CHINESE HAMSTERS WITH SPONTANEOUS DIABETES

By

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ABSTRACT

A qualitative as well as quantitative analysis of testicular tissue was made in 15 rats with alloxan diabetes, 11 Chinese hamsters with spontaneous diabetes, and 6 control animals of each species to determine whether morphological changes also occur in the diabetic test animal. Histometric determinations in both test groups showed a marked decrease in germinal epithelium throughout the testicular tissue and an increase in lumen. The interstitial tissue showed no significant quantitative dissimilarity between healthy and diseased animals. In both animal species we found that the more serious the metabolic disorder was during the period of observation, the more marked were the changes in the germinal epithelium and in the lumen. Qualitatively, disorders of various degrees were found, from a reduced number of sperms and spermatids to severe inhibition of spermatogenesis with cessation of spermatogenesis at the stage of primary spermatocytes. In addition, we observed in both animal species, a marked decline in the number of Leydig cells in cases of severe metabolic disorder. In the diabetic animal as well as in the diabetic human male, the changes in the testes can be determined histologically and both are indicative of hypogonadotrophic hypogonadism frequently encountered in diabetes mellitus.

During the past six years, we were able to demonstrate that sexual disorders in human males with diabetes are the result of hypogonadotrophic hypo-

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gonadism which develops during the course of the metabolic disease (Schöffling 1960, 1965). The condition is characterized by impotence, subnormal size of the prostate gland, reduced sperm count, reduced fructose concentration in the ejaculate, absence of gonadotrophin excretion, changes in urinary steroids, as well as by characteristic histological abnormalities in the testes (Schöffling et al. 1959, 1960, 1961, 1963). On the basis of our histometric determinations in the testicular tissue (Schöffling 1965; Federlin et al. 1965), the changes in the testes can be defined as a general retardation of the spermatogenic process, with partial cessation of spermatogenesis between spermatocytes and spermatids, and thickening of the tunica propria and of the entire interstitial tissue.

The findings obtained in our studies in man prompted us to conduct the experiments in diabetic animals. These are reported as follows. Histological examinations and histometric determinations were performed on the testicular tissue of rats with alloxan diabetes, Chinese hamsters with spontaneous diabetes and were compared with a group of control animals. A test procedure was chosen to answer the question whether the severity of the metabolic disorder and the duration of the diabetic condition were related to the extent of the testicular damage.

Qualitative histological studies of the testes of rats with alloxan diabetes (Jacobs 1937; Goldner & Gomori 1944) were performed by Soulairac et al. (1948) and Hunt & Bailey (1961). Both research teams observed testicular atrophy, subnormal size of the tubules and abnormal spermatogenesis, which in some cases led to complete depopulation with only Sertoli cells remaining. Hunt & Bailey (1961) observed in addition, cessation of secretory activity in the prostate gland and seminal vesicles of alloxan diabetic animals and failure of testicular descent in young animals.

Testicular studies in Chinese hamsters (grey hamster, Cricetulus griseus) have, to the best of our knowledge, not been conducted to date. This animal species was introduced into experimental biology by Hsieh (1919). While breeding a strain for research purposes in tumour pathology, Yerkanian (1958) and Meier & Yerkanian (1959, 1961) observed a metabolic disorder with hyperglycaemia and glycosuria. Further studies revealed that a mutation had occurred during the pairing of siblings, producing spontaneous hereditary diabetes with deterioration of the β-cells of the islets of Langerhans.

MATERIAL AND METHODS

In the present study the histological and histometric examinations of the testicular tissue in 21 rats and 17 Chinese hamsters are described.

(a) Studies in rats: Fifteen animals of a group of 90 rats with alloxan diabetes were selected. All had hyperglycaemia during the entire period of observation but required no insulin treatment for survival.
For analytical purposes, the animals were divided into three groups, those with blood sugar levels of 151–250 mg/100 ml, 251–350 mg/100 ml, and 351–450 mg/100 ml, respectively. The animals were sacrificed after 3, 4, 5, 7, 10, and 12 months. The testes were removed immediately following sacrifice and fixed in Bouin's solution. For further evaluation, the animals were grouped according to length of survival (3–6 months and 7–12 months). Six rats of the same strain and with the same body weight were used as controls, and were sacrificed after 6, 9, and 12 months.

The blood sugars were determined enzymatically according to the method of Huggett & Nixon (1957), and urine sugar values were determined with Clinitest tablets.

(b) Studies in Chinese hamsters: Chinese hamsters of the strain of the C. H. Best Institute of the University of Toronto were chosen: 6 with normal metabolism and 11 with spontaneous diabetes. The Toronto colony was bred from a strain of the Boston species developed by Meier & Yerganian (1959). Our test animals were of the 2nd to 4th generations of the Toronto strain. The diabetic animals were born between 9/17/61 and 6/8/62. Diabetes developed in these animals before the 13th week of life. In 9 of the 11 diabetic hamsters, one of the parents or even both progenitors were also diabetic at the time of our investigation. Insulin treatment was required in 5 of the 11 diabetic animals (test group with «severe metabolic disorder»). In 6 animals, no exogenous insulin was necessary to prevent metabolic decompensation (group with «mild metabolic disorder»). The control group comprised 6 Chinese hamsters of the same strain with no diabetes as indicated by at least 10 tests.

These hamsters were not sacrificed, but, a biopsy of the testes was performed and a piece of tissue the size of a lentil or a pea removed from the medial sections of the right testis. All 11 diabetic test animals were paired 5 times during the 8 weeks preceding biopsy. They produced no offspring in these attempted pairings, whereas normal fertility was present under identical conditions in the control group.

Histological examinations: The tissue sections of the rats and the biopsy material of the hamsters were fixed for more than 6 hours in Bouin's solution (15 parts of aqueous picric acid, 5 parts of concentrated formaldehyde solution, 1 part of glacial acetic acid). This method of fixation, as shown in earlier studies (Federlin & Köster 1954; Federlin et al. 1965), is superior to other procedures used for the examination of testicular tissue and was, therefore, chosen by us.

The tissue was then washed in water and treated further as follows: 24 hours in 70% alcohol (replaced several times), 12 hours in 80% alcohol, 8 hours in 96% alcohol, 16 hours in absolute alcohol (replaced twice). The tissue was then put into methyl benzoate (replaced three times) for 24 hours and into benzene for 1 hour (replaced three times). After transfer into benzene paraffin for 1 hour the pieces of tissue were imbedded in paraffin for 80–96 hours at a thermostatically controlled temperature of 56°C and subsequently imbedded in fresh paraffin. From each paraffin block, 8 random slices of 5 µ thickness were selected and stained with haematoxylin-eosin stain and by the Hoppe technique (haemalum, orange-G-solution, phosphomolybdic acid, aniline blue), as described by Tonutti (1943) and Muschke (1953).

Histological differentiation of the testes of rats was made according to our own definition, based on the work by Leblond & Clermont (1952). Unlike in humans, a cyclic course of spermatogenesis is found in the rat as well as in some other mammals (Leblond & Clermont 1952; Starck 1955; Bargmann 1956). The cycle begins in the centre and ends in the periphery of the testes. Hence, in any section of a tubule, only some of the developmental phases of spermatogenesis are found. The literature distinguishes between as many as 14 phases of spermatogenesis and up to 19 phases.
of spermatohistogenesis. In our study we limited the differentiation to spermatogonia, primary and secondary spermatocytes, spermatids, and immature and mature spermatozoa. This provided us with sufficient information to answer our questions. In addition, Leydig cells in the interstitial tissue of the tubules were examined. When active, Leydig cells have a round and relatively large nucleus and considerable polygonal cytoplasm. When inactive, they are atrophic and can be mistaken for fibrocytes. Fig. 1 shows the normal testicular tissue of a mature white rat. When examining the seminiferous tubule epithelium of Chinese hamsters, the same criteria were used. Here, too, we found a cyclic course of spermatogenesis, i.e., not all the stages of maturation were found side by side in one cross section of a tubule but, instead, these were distributed over the various tubules. The spermatogonia are lined up on the relatively thin basal membrane and appeared as lightly-stained cells with a large amount of cytoplasm and a medium-sized round nucleus, which occasionally shows nucleoli but no definite chromatin structure. The primary spermatocytes are recognized by a nucleus that is occasionally dark brown, with compact chromatin body and sometimes with a considerably larger nucleus containing loose to punctate chromatin elements. Located nearer the lumen are the secondary spermatocytes in the form of pale stained globular cells, smaller than those of the preceding stages of maturation. These are spermatids in the various stages of spermatohistogenesis. The centre of the tubular lumen is filled with mature sperms. Leydig cells with markedly flattened cytoplasm are seen in the septa between adjoining testicular tubules. As in the rat, the nuclei of the Sertoli cells are generally oval structures that are located parallel to the basal membrane and are only rarely in vertical position. Fig. 4 shows the normal testicular tissue of a mature Chinese hamster.

**Histometric determinations:** For a quantitative analysis of the histological sections, we used the Zeiss Integrating Eyepiece I (test point chart) and the point counting method of Hennig (1958). This ocular has a network of 25 points which are arranged within a circle covering the area to be examined during microscopy. Using a method that has recently been described by us (Federlin et al. 1965), the tissue element under a grid point is counted as a »hit«. The sum of the »hits« of each tissue component are then related to the total of all counted »hits«.

In our tests we differentiated between germinal epithelium, lumen, and interstitial tissue. The various developmental stages of spermatogenesis, including Sertoli's cells were considered as germinal epithelium. Those tissue parts which were not attached to the germinal epithelium but were scattered throughout the centre of a tubule were considered as lumen. Interstitial tissue included connective tissue, Leydig cells, tunica propria, as well as the separation which was occasionally caused by shrinkage of tissue during preliminary treatment.

In the testicular tissue of each animal, we counted, at an objective magnification of 10:1, the »hits« in a total of 40 settings (5 settings per slice), i.e., 1000 »hits« were counted per animal. For this purpose, the settings were arbitrarily distributed over each section.

The accuracy of our evaluation was determined by means of nomograms which had been worked out for the Integrating Eyepiece I. The statistical analysis of this histometric examination was recently explained by us in detail (Federlin et al. 1965) and need not be further elaborated upon. The subjective error was between ±2.6 and ±3.55 %, and the objective error was constantly below 1.1 %. The mean values of our histometric determinations in comparative series were statistically determined by means of Student's t test. A difference between two mean values was considered significant when \( P < 0.001 \).
RESULTS

(a) Studies in rats: We found an insignificant decrease in the number of mature sperms, spermatids, and secondary spermatocytes in the testicular tissue of rats with mild alloxan diabetes (mean blood sugar value between 151 and 250 mg/100 ml). Primary spermatogonia and Leydig cells appeared unchanged.

In animals with moderately severe diabetes (mean blood sugar value between 251 and 350 mg/100 ml) we observed that the germinal epithelium had somewhat deteriorated. Compared with normal testicular tissue (Fig. 1), the numbers of secondary spermatocytes, spermatids, immature spermatozoa and sperms were markedly reduced. The secondary spermatocytes were occasionally detached and found in the lumen. The remaining germinal epithelium and Leydig cells appeared to be only slightly changed (Fig. 2).

In rats with severe diabetes mellitus (mean blood sugar values between 351 and 450 mg/100 ml) only a few of the testicular tubules still showed normal structure of the germinal epithelium. Most of the tubules contained only a few spermatogonia and primary spermatocytes. Giant cells containing several nuclei were occasionally found, an observation also made by Cohrs et al. (1958) in rats with degenerative testicular changes. Spermatozoa and spermatids were no longer observed. Occasionally, the tubules contained only a reduced number of spermatogonia. In this test group Leydig cells were largely absent (Fig. 3).

The histometric determination in 6 healthy rats showed that the germinal epithelium made up 70.9 % and the lumen 8.65 % of the overall testicular tissue. In the group of rats with alloxan diabetes, on the other hand, the percentage of germinal epithelium had decreased to 62.2 % while that of the

Fig. 1.
Normal testicular tissue of a mature white laboratory rat. HOPA staining. × 166.
Fig. 2.
Testicular tissue of an alloxan diabetic rat with moderately severe diabetes. Reduced amount of germinal epithelium. Premature shedding of secondary spermatocytes from the germinal epithelium into the lumen. Spermatids and sperms are largely absent. HOPA staining. × 166.

Fig. 3.
Testicular tissue of an alloxan diabetic rat with a severe metabolic disorder. Almost complete loss of germinal epithelium, except for a thin layer of spermatogonia. Haematoxylin-eosin stain. × 166.

lumen had increased to 18.2%. These differences are statistically significant. The interstitial tissue had slightly decreased from 20.45% to 19.6% (Table 1, Section A).

When looking for an interrelation between duration of the metabolic disorder and magnitude of testicular changes in the rat (Table 1, Section B), no
Table 1.

Results of histometric determination in testicular tissue of rats.
A. Comparison between healthy and alloxan diabetic rats.
B. Comparison between diabetic rats subjected to the test for varying periods of time.
C. Comparison between diabetic rats with a metabolic disorder varying in severity.
(Mean values and range of deviations in per cent).

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<th>Interstitial tissue</th>
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<tr>
<td>A.</td>
<td></td>
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<tr>
<td>(1)</td>
<td>Healthy rats</td>
<td>70.90 ± 0.91</td>
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<td>62.20 ± 2.08</td>
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<td></td>
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<td>B.</td>
<td></td>
<td></td>
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<td>Diabetic rats</td>
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<td></td>
<td>P</td>
<td>&gt; 0.1</td>
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<tr>
<td>C.</td>
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<tr>
<td></td>
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<td>PC 2–3</td>
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significant differences between groups under observation for 3–6 months and 6–12 months were observed.

The effect of the severity of the diabetic condition on the magnitude of testicular changes in the rats examined is shown in Section C of Table 1. The percentage of germinal epithelium is 64.7 % in the group with a mild metabolic disorder, 61.5 % in the group with a moderately severe metabolic disorder, and 60.2 % in the group with a severe metabolic disorder. With regard to the lumen, the following values were obtained: 15.4 %, 19.2 %, and 20.1 %, respectively. The percentages for the interstitial cells were 19.9 %, 19.3 %, and 19.7 %.

(b) Studies in Chinese hamsters: In Chinese hamsters with a mild metabolic
disorder not requiring insulin, decreased germinal epithelium in addition to an increased number of immature structures in the lumen we found. Compared with normal testes (Fig. 4), it was in particularly the number of sperms, spermatids and secondary spermatocytes that were reduced. The remaining developmental stages as well as Leydig cells appeared to be normal. The amount of basal membrane had also decreased in some animals of this group (Fig. 5).

**Fig. 4.**
Normal testicular tissue of mature Chinese hamster. Haematoxylin-eosin stain. × 166.

**Fig. 5.**
Testicular tissue of a Chinese hamster with spontaneous diabetes of moderate severity. Seminiferous epithelium is slightly reduced. Number of spermatids and sperms is markedly decreased. PAS stain. × 166.
Complete cessation of spermatogenesis at the stage of primary spermatocytes was observed in these Chinese hamsters with severe diabetes requiring treatment with insulin. However, in some of the tubules of these animals, we could barely observe a thin layer of occasional spermatogonia and Sertoli cells at the basal membrane. The number of Leydig cells was also markedly reduced and the tunica propria was thickened sporadically (Fig. 6).

In the histometric analysis, the percentage of germinal epithelium was 77% in the healthy hamster and only 64.4% in the diabetic animal. The percentage of lumen was 5.2% in the healthy and 12.5% in the diabetic animals. These differences are statistically significant. The percentage of interstitial tissue had increased from 17.8% in healthy animals to 20.1% in those with diabetes (Table 2, Section A).

The germinal epithelium comprised 70.6% of the overall testicular tissue in the group with a mild metabolic disorder and 63.5% in the group with severe diabetes requiring treatment with insulin. With regards to the lumen, we determined 10.4% in the first and 15.0% in the second group. The differences in germinal epithelium and lumen are substantial, whereas no definite variations were obtained regarding the interstitial tissue: 19.9% as compared to 21.5% (Table 2, Section B).

*Fig. 6.*

Testicular tissue of a Chinese hamster with spontaneous diabetes of moderate severity. In the centre, canals with complete cessation of spermatogenesis at the stage of primary spermatocytes or spermatogonia. The cut tubule also shows markedly reduced seminiferous epithelium, which, at this point, still contains, however, primary and secondary spermatocytes, but no spermatids and sperms. HOPA stain. $\times$ 166.
Results of histometric determination in testicular tissue of Chinese hamsters.

A. Comparison between healthy and diabetic hamsters.

B. Comparison between diabetic hamsters with a mild metabolic disorder and those with severe diabetes ascribed to insulin deficiency.

(Mean values and range of deviations in per cent).

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<td>A. (1) Healthy hamsters</td>
<td>77.00 ± 0.83</td>
<td>5.20 ± 0.23</td>
<td>17.80 ± 0.89</td>
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<td>A. (2) Diabetic hamsters</td>
<td>67.40 ± 4.96</td>
<td>12.50 ± 3.24</td>
<td>20.10 ± 2.45</td>
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<td>&lt; 0.0005</td>
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B. (1) Diabetic hamsters Moderate metabolic disorder

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<th>Lumen</th>
<th>Interstitial tissue</th>
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<tbody>
<tr>
<td>(2) Diabetic hamsters Severe metabolic disorder</td>
<td>63.50 ± 4.92</td>
<td>15.00 ± 2.88</td>
<td>21.50 ± 2.79</td>
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<tr>
<td>P</td>
<td>&lt; 0.01</td>
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DISCUSSION

The object of the present study was to determine the histological changes occurring in the testes of rats with alloxan diabetes and of Chinese hamsters with spontaneous diabetes. In both species, we found interference with the spermatogenesis that was dependent on the severity of the metabolic disorder. By means of histometric analysis, it was possible to determine that the percentage of »germinal epithelium« was significantly reduced in diabetic rats and hamsters and that that of the »lumen« was correspondingly increased. With regard to the »total interstitial tissue«, no significant differences were observed between controls and diabetic rats or hamsters. The qualitative analysis also showed a definite decrease of Leydig cells in animals with a severe metabolic disorder.

In similar histometric tests with testicular tissue of diabetic men (Schöffling 1965; Federlin et al. 1965), we observed an even more marked atrophy of the germinal epithelium. Here the percentage had decreased from 69.1% in the control group to 52.0% in the diabetic group, and the »proportion of the lumen« had moderately and that of the total interstitial tissue markedly increased.

Our tests in rats with alloxan diabetes thus confirm and extend the observations of Soulairac et al. (1948) as well as of Hunt & Bailey (1961). In Chinese
hamsters, in which degeneration of the B-cells and insulin deficiency diabetes occur spontaneously rather than induced by alloxan, we were able, for the first time, to demonstrate testicular changes which largely simulate those in alloxan diabetic rats.

Severe functional disorders in the reproductive glands and serious changes in the testes also occurred in rats which developed diabetes mellitus after a 95% pancreatectomy (Foglia et al. 1963). In the course of this study, attempts at mating were made which indicated a decrease in fertility by one third. Fertility disorders and changes in the reproductive glands also occur in mice with an «obese-hyperglycaemic syndrome» inherited as a recessive characteristic (Lane 1959). Here the atrophy of Leydig cells is more predominant, however, than the decrease in germinal epithelium (Hellman et al. 1963). Testicular atrophy or aspermia has also been observed in pancreatectomized cats (Kraus 1921) and roosters (Belkin et al. 1931; Lepkovsky et al. 1964).

On the basis of our own findings and those of other investigators, it follows that functional disorder of the reproductive gland and testicular atrophy with hypospermatogenesis and partial cessation of spermatogenesis occur in the diabetic test animals as well as in the diabetic human male. Whereas in animals with a severe metabolic disorder, the lumen of the testicular tissue increased, total interstitial tissue remained the same, and the number of Leydig cells was reduced. In the diabetic test animal as well as in the diabetic human male, hypogonadotrophic hypogonadism occurs in the course of the metabolic disease.

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