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Immunohistochemical distribution of sulfhydryl oxidase in the human testis

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Summary. Sulfhydryl oxidase (SOx) is an enzyme that catalyzes the oxidation of sulfhydryl compounds. It is present in mitochondria of certain testicular cells at specific stages of functional activation. In the mature human testis moderate SOx immunoreactivity is found in Leydig cells, and lacking in Sertoli and in peritubular cells. The A_{dark} spermatogonia usually contain immunoreactive mitochondria, while in A_{pale} spermatogonia immunoreactivity is mostly low. In stage V of spermatogenesis, A_{pale} spermatogonia were found containing immunoreactive material. Leptotene (stages IV and V) and zygotene (stage VI) primary spermatocytes display a moderate immunoreaction. It is strongest in pachytene spermatocytes of stages I–IV, decreases in stage V, and is low during diakinesis and in secondary spermatocytes. Late spermatids usually show a stronger immunoreactivity than early spermatids. At stage V of spermatogenesis the late spermatids contain only few immunoreactive particles. Spermatozoa are free of SOx-immunoreactive mitochondria. In residual bodies small amounts of SOx-immunoreactive particles are seen. Compared to rat and hamster testis, SOx immunoreactivity of the human testis is less clearly stage-dependent and it is not confined to certain germ cell stages. As deduced from the findings in patients with spermatogenic disorders, the SOx immunoreactivity of spermatogonia in human testis seems to be of diagnostic relevance.

Key words: Testis – Spermatogenesis – Leydig cells – Sulfhydryl oxidase – Man

Sulfhydryl oxidase (SOx, or thioloxidase, EC 1.8.3.2.) is an enzyme that catalyzes the oxidation of sulfhydryl compounds such as glutathione, cysteine or thioglycerol, utilizing molecular oxygen as electron acceptor. The newly formed disulfide bonds are thought to result in conformational changes of membrane proteins, e.g., in spermatozoa (Haugaard 1968) that are required for certain membrane-associated processes. A relatively high level of SOx activity in hamster epididymal fluid was reported by Chang and Morton (1975) and in rat seminal vesicle secretion by Chang and Zirkin (1978). The rat seminal enzyme was purified and characterized by Ostrowski et al. (1979) and by Ostrowski and Kistler (1980). We have prepared an antibody against rat seminal sulfhydryl oxidase (Seitz et al. 1988), which localizes the antigen in the halo of rat seminal vesicle secretory vacuoles. Using the same immuno-electron-microscopic approach, we were able to detect a cross-reactive species of the antigen present in the matrix of mitochondria in several tissues such as striated ducts of salivary glands, distal tubules in the kidney, and pachytene spermatocytes of the rat and hamster testis (Seitz et al. 1988).

More detailed immunohistochemical studies in the testis of rat, juvenile (Seitz et al. 1990) and adult (Kumari et al. 1990), of hamster, juvenile (Bergmann et al. 1991) and adult (Kumari et al. 1990), as well as in *Ambystoma mexicanum* (Oehmen et al. 1991) have shown that SOx is expressed in a stage-dependent manner during development of spermatogenesis, involution and recrudescence (Bergmann et al. 1990) and during normal intact spermatogenesis. SOx immunoreactivity is strongest in mitochondria of pachytene spermatocytes. Our observations suggest that SOx is another example of differential gene activation associated with a developmental process in gametes, similar to stage-dependent expression of LDH-X and cytochrome C (Hintz and Goldberg 1977; Wheat et al. 1977). The present study provides information on SOx distribution during normal spermatogenesis in the human testis. As has been recently shown (Meinhardt et al. 1991), the specific expression pattern of the enzyme at certain stages of spermatogenesis allows its use as a functional marker of spermatogenic cells both in normal and in pathologically altered spermatogenesis.

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Table 1. Stages of spermatogenesis and SOx expression. ∅, Negative; ±, background level; +, positive; ++, strong; +++, very strong; rb, residual bodies

Testicular cell type		I	II	III	IV	V	VI
Spermatozoa			∅				
Spermatids	late	++	+(rb)			++	++
	middle			+++	++/+++		
	early	+	+				
II. Spermatocytes							++
I. Spermatocytes	-Diakinesis						+
	-Pachytene late			+++	++/+++	++	
	early, mid	++	+++				
	-Zygotene						+
	-Leptotene			±	+	+	
Spermatogonia							
	B	∅	∅	∅			
	A_{pale}	+	+	+	+	+	+
	A_{dark}	+	+	+	+	+	+
Sertoli cells		±	±	±	±	±	±
Peritubular cells		∅	∅	∅	∅	∅	∅
Leydig cells		+	+	++	+	+	+

Materials and methods

Antibody

Antigen isolation and antibody preparation were performed as previously described (Seitz and Aumüller 1989; Kumari et al. 1990). The antibody was monospecific in immunoblotting test. A testis biopsy sample was homogenized in TRIS/HCl buffer (50 mM, pH 8.0) containing 150 mM NaCl and 5 mM EDTA and centrifuged for 60 min. Rat seminal vesicle tissue was similarly treated. Supernatants were diluted with sample buffer (final protein concentration 9 mg/ml) and subjected to SDS-PAGE according to the method of Laemmli (1970). Proteins were transferred electrophoretically onto nitrocellulose according to the method of Towbin et al. (1979) using a Biorad transblot cell. Nitrocellulose sheets containing transblotted proteins were incubated with the antiserum and the immunoreaction was visualized as previously described (Kumari et al. 1990).

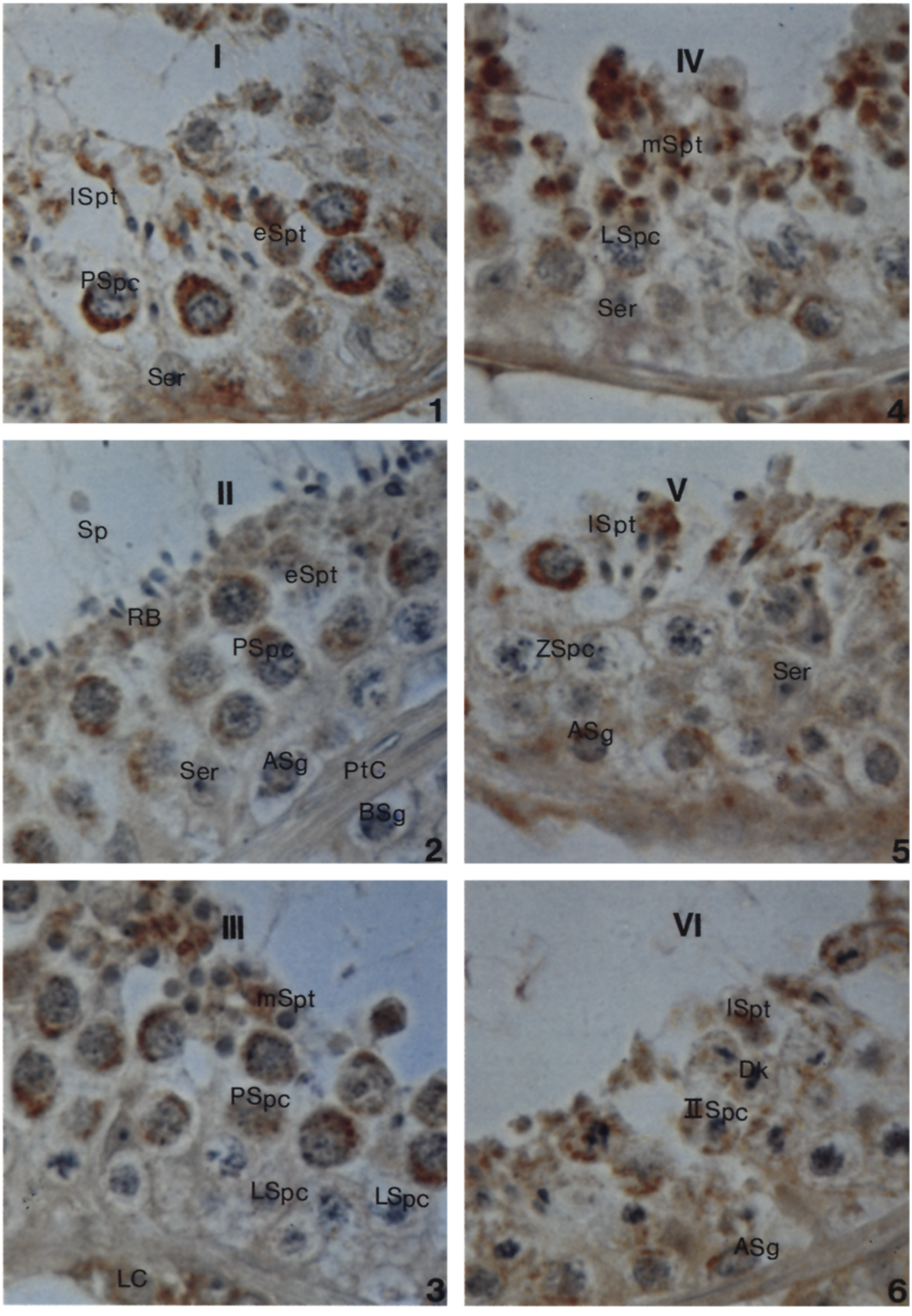
Tissue

Bouin-fixed testicular tissue samples from 3 men (aged 27, 69 and 73 years, respectively) taken at routine autopsies (Department of Pathology, Philipps University Marburg; courtesy of Professor Thomas) and from one 47-year-old man (sexual delinquent who had undergone voluntary castration; specimen kindly provided by Professor W. Schulze, Department of Anatomy, University of Hamburg) were used for paraffin sections cut at 5 µm thickness. Testicular biopsies, taken at the Andrology Unit, Department of Dermatology, Philipps University Marburg (Professor W. Krause) fixed in low-glutaraldehyde (0.1%) solutions and embedded in LR-White and in Epon were also tried. Semithin sections, however, were found unsuitable, since the immunoreaction was apparently blocked by the embedding medium.

Incubation procedure

Paraffin sections were deparaffinized, passed through alcohols into water and then immersed in phosphate-buffered saline (PBS). Pretreatment with 3% H_2O_2 was performed for 30 min and with 10% bovine serum albumin (BSA) for 15 min. Sections were then incubated with the SOx antibody overnight at 4° C at a dilution of 1:200. After several rinses with PBS, sections were incubated for 30 min at room temperature with swine anti-rabbit IgG (1:150, Dako), washed thoroughly with PBS and then treated with the soluble PAP complex (1:200, Dako) for 20 min at room temperature. Enzyme activity was developed by incubation with H_2O_2-solution (7 µl/100 ml TRIS-HCl buffer 0.1 M, pH 7.6) containing 10 mg diaminobenzidine (Fluka, Switzerland). After rinsing, sections were either briefly counterstained with hemalum or immediately dehydrated, mounted in resin and coverslipped. Control experiments included incubation of rat testis sections with the SOx antibody (positive control) or replacement of the SOx antibody

Figs. 1–6. Immunoreactivity patterns of sulfhydryl oxidase during different stages (*I–VI*) of human spermatogenesis. ×380. **Fig. 1.** Stage *I*: strong reaction in pachytene spermatocytes (*PSpc*), moderate reaction in late spermatids (*lSpt*), weak reaction in early spermatids (*eSpt*), background reaction in Sertoli cells (*Ser*). **Fig. 2.** Stage *II*: strong reaction in pachytene spermatocytes (*PSpc*), moderate reaction in early spermatids (*eSpt*), A spermatogonia (*ASg*) and residual bodies (*RB*), no reaction in Sertoli cells (*Ser*), peritubular cells (*PtC*)) and B spermatogonia (*BSg*). **Fig. 3.** Stage *III*: strong reaction in pachytene spermatocytes (*PSpc*) and mid spermatids (*mSpt*), moderate reaction in Leydig cells (*LC*) and uncertain reaction in intermediary or leptotene spermatocytes (*LSpc*). **Fig. 4.** Stage *IV*: strong to moderate reaction in mid spermatids (*mSpt*), weak reaction in leptotene spermatocytes (*LSpc*). **Fig. 5.** Stage *V*: moderate reaction in late spermatids (*lSpt*) and A spermatogonia (*ASg*), weak reaction in zygotene spermatocytes (*ZSpc*). **Fig. 6.** Stage *VI*: moderate reaction in late spermatids (*lSpt*) and secondary spermatocytes (*IISpc*), weak reaction in diakinesis of primary spermatocytes (*Dk*) and A spermatogonia (*ASg*)


I
lSpt
eSpt
PSpc
Ser
1
II
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eSpt
RB
PSpc
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ASg
PtC
BSg
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III
mSpt
PSpc
LSpc
LSpc
LC
3
IV
mSpt
LSpc
Ser
4
V
lSpt
ZSpc
Ser
ASg
5
VI
lSpt
Dk
IISpc
ASg
6

by an irrelevant antibody such as anti-prostatic acid phosphatase or by 2% swine serum in PBS (negative control). Photographs were taken with a Zeiss Axiomat photomicroscope.

Results

Immunohistochemistry of testicular sulfhydryl oxidase

The classification of Clermont (1963) is used in the present report for the different stages of human spermatogenesis. A survey on the distribution of SOx immunoreactivity in human testis is given in Table 1. A consistent finding was the positive immunoreactivity of interstitial Leydig cells, the absence of an immunoreaction from peritubular cells and the low expression of SOx immunoreactivity in Sertoli cells. In spermatogenic cells SOx immunoreactivity was found in A_{dark} and A_{pale}, but not in B spermatogonia. In primary spermatocytes it became apparent already in leptotene, reached its maximum in mid pachytene and decreased in late pachytene. In secondary spermatocytes the immunoreaction was stronger than in early spermatids. It increased in mid and late spermatids and was still visible in residual bodies after spermiation. Mature spermatozoa did not contain immunoreactive material.

The characteristic distributional pattern resulted in typical figures during the individual stages of spermatogenesis (Figs. 1–6). In stage I, the most prominent immunoreactive cells were the early pachytene spermatocytes and late spermatids (Fig. 1). In stage II, mid pachytene spermatocytes were labeled and an adluminal rim of intermediate immunoreactivity was found in residual bodies (Fig. 2). In stage III, basally located A spermatogonia, mid pachytene spermatocytes and mid spermatids were labeled (Fig. 3). Adluminal concentration of strong immunoreactivity was characteristic of stage IV, where late pachytene spermatocytes and mid spermatids are present (Fig. 4). This arrangement pattern was most readily recognized in survey magnifications. In stage V, leptotene spermatocytes with low immunoreactivity were more prevalent and the immunoreactivity of late spermatids had decreased (Fig. 5). In stage VI, the immunoreactivity of the diakinesis spermatocytes was marginally concentrated, that of the zygotene spermatocytes was stronger (Fig. 6).

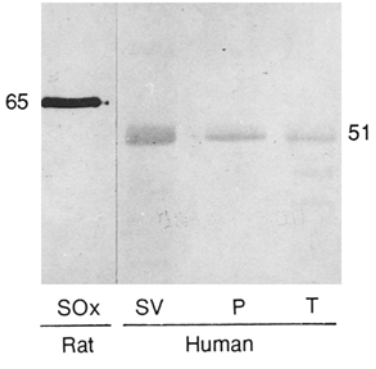

Fig. 7. Western blotting of extracts from rat seminal vesicle secretion (*SOx Rat*) and human seminal vesicle (*SV*), prostate (*P*) and testis (*T*). The antibody against rat seminal vesicle SOx (M_r 65 kD) is cross-reactive with an human antigen of 51 kD

Antibody specificity

Immunoblot analysis of our antibody using SDS-gel electrophoresis followed by Western blotting resulted in two immunoreactive bands with the tissue extracts. With the rat seminal vesicle extract the band was in the molecular weight range of 65 kDa, while with human prostate, seminal vesicle and testis extracts, the molecular weight of the immunoreactive protein was significantly lower, ranging at 51 kDa. Also, the immunoreaction was less strong (Fig. 7).

Discussion

Antibody specificity

With regard to the fact that the SOx antigen for the present antibody is derived from the rat seminal vesicle secretion, the question of antibody specificity is of particular importance. As has been shown by immunoblotting of human testicular prostatic and vesicular tissue extracts, the antibody recognizes a protein of 51 kDa apparent molecular weight. This value is below that of the rat secretory protein which is 65 kDa. Obviously, the antibody recognizes a non-secretory non-tissue-specific, non-species-specific membrane- or particle-bound protein. As shown in our previous work (Seitz et al. 1988) in the seminal vesicles, mitochondria are immunoreactive with the SOx antibody.

Expression in mitochondria

The appearance of immunoreactive SOx in germ cells seems to be related with certain functional stages of mitochondria. During human spermatogenesis (for ultrastructure, see Holstein and Roosen-Runge 1981), clusters of mitochondria are found in A spermatogonia and in pachytene spermatocytes. Mitochondria of pachytene spermatocytes often have extended intercristal spaces as a salient morphological feature. The observed (1) increase of SOx immunoreactivity in meiotic cells, (2) its distributional pattern in spermatocytes and spermatids, (3) the aggregation and condensation of immunoreactive particles in late spermatids, and (4) the sequestration via residual bodies (see Breucker et al. 1985) are clearly in favour of a mitochondrial localization of the immunoreactivity.

It is strikingly similar to that of LDH-X, which also has been shown to be located within the mitochondrial matrix (Blanco et al. 1975).

Using a histochemical approach, Hintz and Goldberg (1977) reported the presence of LDH-X only at mid-pachytene stage onwards. With more refined and better techniques, Li et al. (1989) were able to detect LDH-X

synthesis at an earlier stage (preleptotene spermatocytes), although at a much lower level. SOx immunoreactivity was also seen in human leptotene spermatocytes and it increased substantially at mid pachytene.

Our attempts of an ultrastructural localization have been unsatisfactory. Although the antigen is not especially fixation sensitive, the embedding procedure of low-glutaraldehyde-fixed, non-osmium-treated tissue, even in low temperature resins, was absolutely detrimental to SOx immunoreactivity. Immunolabeling experiments with ultracryosections are underway.

The functional role of the mitochondrial sulfhydryl oxidase is completely unknown as yet. Siliprandi et al. (1974) changed the ratio of sulfhydryl groups (-SH) to disulfides (S-S) by use of the oxidant diamide, and reversed the reaction by use of DTE (dithioerythritol) within isolated mitochondria. They could demonstrate that important mitochondrial functions, e.g., anion and cation transport, ATPase activity, swelling and contraction processes and coupling mechanisms may be regulated via changes in this ratio. If sulfhydryl oxidase is involved in such a mechanism, its expression in certain forms of human spermatogenic cells would be of major functional significance for these cells.

Expression pattern in testicular cells

In the human testis, strong immunoreactivity was found in somatic testicular cells, such as Leydig cells, and in germ cells (A spermatogonia, spermatocytes, mit spermatids). It was absent from peritubular cells, B spermatogonia and mature spermatozoa. Other forms, e.g., Sertoli cells and early spermatids had a weak to uncertain immunoreaction. Strongest immunoreactions were seen in late pachytene spermatozoa during stages III and IV, and middle spermatids. Although the immunoreaction was less in subsequent stages of the respective cells, the mechanism for reduction (phagocytosis of mitochondria? formation of new mitochondria?) is not clear. The same is true for the reduction of immunoreaction in residual bodies, which is significantly lower than, e.g., in residual bodies of rat testis.

The immunoreaction pattern of SOx in the human testis is surprisingly similar to that found in the developing rat testis. This may be due to the specific arrangement of spermatogenic cells in human testis, which considerably differs from that in other animals (see Schulze et al. 1986) and may require highly regulated local paracrine interactions. In rat testis of 5- to 30-day-old animals, SOx immunoreactivity was seen in A spermatogonia, where it was aggregated on one side of the cell, while no immunoreactivity was present in intermediate and B spermatogonia (Seitz et al. 1990). In Sertoli cells from 5-day-old animals, there was a positive but diffuse staining. From day 10 onwards, a slight aggregation of immunoreactivity was observed, which was concentrated basally on day 20 and disappeared by day 26.

Another consistent finding was the expression of SOx immunoreactivity in human Leydig cells. This was also typical for rat prespermatogenesis (Seitz et al. 1990), as well as for hamster prespermatogenesis (Bergmann et al. 1990). The distribution of immunoreactivity in Leydig cells is more homogeneous, e.g., than in pachytene spermatocytes and is apparently not particle-bound. It is uncertain whether the immunoreactivity is related to the mitochondria in this cell type.

Taking together our findings on SOx immunoreactivity in the human testis, we conclude that its presence in A spermatogonia, in pachytene spermatocytes and in elongating spermatids render it an important marker for germ cell multiplication, onset of meiosis and spermatid differentiation in the human testis.

References

Bergmann M, Kumari M, Aumüller G, Hoffmann K, Seitz J (1990) Distribution pattern of testicular sulfhydryl oxidase (SOx) immunoreactivity in the Djungarian hamster (*Phodopus sungorus*) during photoperiodically induced involution and recrudescence. Int J Androl 13:488–499

Bergmann M, Nashan D, Steinmeyer E, Aumüller G, Seitz J, Nieschlag E (1991a) Das Verteilungsmuster der testikulären Sulfhydryloxidase. Immunreaktivität in menschlichem Biopsiematerial. Anat Anz (in press)

Bergmann M, Oehmen F, Kumari M, Aumüller G, Seitz J (1991b) Immunohistochemical localization of testicular sulfhydryl oxidase (SOx) during sexual maturation of the Djungarian hamster (*Phodopus sungorus*). J Reprod Fertil 91:259–265

Blanco A, Zinkham WH, Walker DG (1975) Cellular localization, catalytic properties, and genetic control of synthesis. In: Markert CL (ed) Isozymes: developmental biology, vol 3. Academic Press, New York, pp 297–312

Breucker H, Schäfer E, Holstein AF (1985) Morphogenesis and fate of the residual body in human spermiogenesis. Cell Tissue Res 240:303–309

Chang TSK, Morton B (1975) Epididymal sulfhydryl oxidase: a sperm-protecting enzyme from the male reproductive tract. Biochem Biophys Res Commun 66:309–315

Chang TSK, Zirkin BR (1978) Distribution of sulfhydryl oxidase activity in the rat and hamster male reproductive tract. Biol Reprod 17:745–748

Clermont Y (1963) The cycle of seminiferous epithelium in man. Am J Anat 112:35–45

Haugaard N (1968) Cellular mechanism of oxygen toxicity. Physiol Rev 48:311–373

Hintz M, Goldberg E (1977) Immunohistochemical localization of LDH-X during spermatogenesis in mouse testes. Dev Biol 57:375–384

Holstein AF, Roosen-Runge EC (1981) Atlas of human spermatogenesis. Grosse, Berlin

Kumari M, Aumüller G, Bergmann M, Meinhardt A, Seitz J (1990) Stage-dependent appearance of sulfhydryl oxidase during spermatogenesis in the testis of rat and hamster. Histochemistry 94:365–371

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Li SSL, O'Brien DA, Hou EW, Versola J, Rockett DL, Eddy EM (1989) Differential activity and synthesis of lactate dehydrogenase isozymes A (muscle), B (heart), and C (testis) in mouse spermatogenic cells. Biol Reprod 40:173–180

Meinhardt A, Bergmann M, Seitz J, Aumüller G (1991) Verteilungsmuster der Sulfhydryloxidase-Immunreaktivität während der normalen und pathologisch veränderten Spermatogenese des Menschen. Dreiländertagung "Fertilität und Sterilität", Goslar, May 29–June 1 1991. Alete Wissenschaftlicher Dienst, München

Oehmen F, Aumüller G, Bergmann M, Seitz J (1991) Distribution

of sulfhydryl oxidase (SOx) immunoreactivity in the testis of the axolotl (*Ambystoma mexicanum*, Amphibia, Urodela). Tissue Cell 23:377–384

Ostrowski MC, Kistler WS (1980) Properties of a flavoprotein sulfhydryl oxidase from rat seminal vesicle secretion. Biochemistry 19:2639–2645

Ostrowski MC, Kistler WS, Williams-Ashman HG (1979) Purification and cell-free synthesis of a major protein from rat seminal vesicle secretion. J Biol Chem 254:383–390

Schulze W, Riemer M, Rehder U, Höhne KH (1986) Computer-aided three-dimensional reconstructions of the arrangement of primary spermatocytes in human seminiferous tubules. Cell Tissue Res 244:1–8

Seitz J, Aumüller G (1989) Secretory proteins from male accessory sex glands: Isolation, biochemical and functional characterization. In: Holstein AF, Voigt KD, Grässlin D (eds) Reproductive biology and medicine. Diesbach, Berlin, pp 112–118

Seitz J, Keppler C, Bergmann M, Aumüller G (1988) Thiol oxidase – a new mitochondrial enzyme: characterization and immunocytochemical localization. Eur J Cell Biol 46 [Suppl 22]:69

Seitz J, Kumari M, Aumüller G (1990) Sulfhydryl oxidase – an immunohistochemical marker for postnatal differentiation of rat testis. Anat Rec 226:91A

Siliprandi D, Scutari G, Zoccaroto F, Siliprandi N (1974) Sulfhydryl group oxidation and mitochondrial function. In: Azzone GF, Klingenberg ME, Quaglioviello E, Siliprandi N (eds) Membrane proteins in transport and phosphorylation. Elsevier, Amsterdam, pp 265–274

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. Proc Natl Acad Sci USA 76:4350–4354

Wheat E, Hintz M, Goldberg E, Margoliash E (1977) Analyses of stage-specific multiple forms of lactate dehydrogenase and of cytochrome c during spermatogenesis in the mouse. Differentiation 9:37–41