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EXPERIMENTAL STUDIES ON CONNECTIVE TISSUE OF THE CAPSULAR LIGAMENT

Influences of Aging and Sex Hormones

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Influences of aging and sex hormones on connective tissue metabolism in the hip joint capsule of Wistar rats were analyzed both biochemically and morphologically. As age advanced, collagen content and collagen fibril diameter of the hip joint capsule tended to increase gradually in both sexes until sexual maturation was reached. Collagen content was significantly greater in males than in females after sexual maturation. The collagen content and fibril diameter were considerably increased by ovariectomy and significantly decreased by the administration of estrogen, or estrogen combined with progesterone, whereas they were significantly increased by the administration of testosterone in orchiectomized male rats.

Key words: hip joint capsule; aging; sex hormones; colagen content; collagen fibril diameter

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The most prominent clinical sign of congenital dislocation of the hip in the perinatal period is relaxation of the hip joint capsule. It has been suggested by Andrén & Borglin (1961 a, b) that female sex hormones participate in the relaxation.

The aim of the present experiment is to study the influences of sex hormones on collagen metabolism in the joint capsule by analyzing the collagen content and collagen fibril diameter. This analysis is an approach to clarifying the causative factors of the relaxation.

MATERIALS AND METHODS

Treatment of animals

Fifty-two Wistar rats, 26 females and 26 males, 3–36 weeks of age, were subjected to an analysis of the influences of aging and 180 Wistar rats, 105 females and 75 males, were subjected to an analysis of the influences of sex hormones.

The rats were kept in individual cages and fed on Nihon CLEA Rat Chow and given water *ad libitum*. They underwent ovariectomy, orchiectomy or sham operation at 3 weeks of age with or without subsequent hormone treatment.

The hormones administered to each animal group were estradiol benzoate (20 μg, per rat), progesterone (10 mg, per rat) and testosterone propionate (2.5 mg, per rat) dissolved in 0.1 ml of sesame oil. Control groups received sesame oil only. The injections were given twice a week subcutaneously.

At 10 weeks of age, the animals were first stunned with a blow and then killed by cervical dislocation. The joint capsules of the hip and knee were dissected free of fatty tissue; the right capsules were prepared for electron microscopic examination and the left ones were hydrolyzed and assayed for hydroxyproline.

Measurement of collagen fibril diameter

After dissection, the specimens were fixed in 1 per cent osmium tetroxide solution in 0.2 M veronal acetate buffer, pH 7.4, dehydrated through a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut with glass-knives on an ultramicrotome (Porter-Blum MT-2). The sections were stained with uranyl acetate and examined under the electron microscope (Hitachi HS-7D type) with an accelerated voltage of 50 KV and a direct magnification of 30,000.

Four areas in every section were selected at random and three arbitrarily selected portions of each area were photographed. Collagen fibril diameter was determined by calculating an average value for 300 fibrils obtained on twelve randomly selected visual fields. Standard polystyrene particles with a constant diameter (910 Å) were always photographed under the same conditions as were used for the examination of tissue specimens.

Analysis of collagen content

The determination of collagen content was performed according to the method reported by Woessner in 1961. The specimens were weighed immediately after dissection, hydrolyzed with 6N-HCl and neutralized with 2.5N-NaOH. The samples were centrifuged at 3,000 rev/min for 5 minutes, after removing impurities with an equivalent mixture of activated charcoal and Dowex 1. The supernatants and hydroxyproline standard solution (100 μg/ml) were oxidized with chloramine T which was then destroyed by perchloric acid. After paradimethylaminobenzaldehyde solution was added and the tubes shaken, they were placed in a 60° C water bath for 20 minutes and then cooled in tap water for 5 minutes. After the developed color became stable, the absorbancy of the solutions was determined spectrophotometrically at 557 mμ.

Collagen content was expressed as the amount of hydroxyproline measured.

All calculations were subjected to statistical analysis using Student's *t* test.

RESULTS

Collagen fibril diameter

The collagen fibril diameter of the hip joint capsule tended to increase gradually

Table 1. Influence of age and sex on collagen fibril diameter of hip joint capsule in Wistar rat.

Age (weeks)	Fibril diameter (Å) Male	Female
3	409.1 ± 60.6 (6)	380.9 ± 55.7 (6)
5	514.4 ± 25.4 (7)	476.1 ± 54.9 (7)
10	535.7 ± 65.2 (5)	503.2 ± 73.8 (5)
20	553.6 ± 44.3 (5)	509.5 ± 57.9 (5)
36	571.4 ± 50.0 (3)	522.9 ± 67.3 (3)

Values are presented as means ± s.e.; the number of animals is given in parentheses.

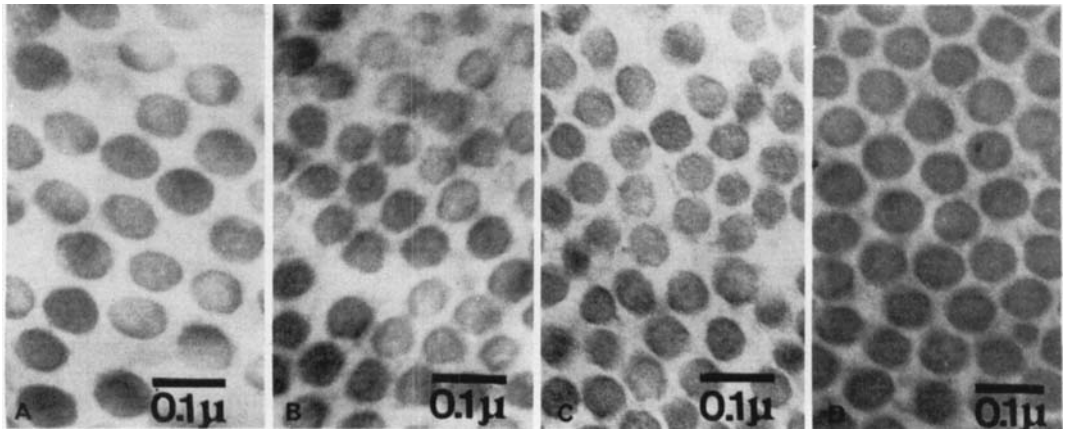

Figure 1. Electron microscopic findings of collagen fibrils of hip joint capsules in 10-week-old ovariectomized Wistar rats with or without hormone treatment. (A): not treated, (B): treated with estrogen, (C): treated with a combination of estrogen and progesterone, (d): treated with progesterone.

Table 2. Effect of gonadectomy and hormone replacement on collagen fibril diameter of hip joint capsule.

Exp. group	No. of animals	Body wt. (g)	Fibril diameter (Å)
1. Sham OVX	8	158 ± 15	533.6 ± 71.3
2. OVX	16	169 ± 15	559.5 ± 86.1
3. OVX+EB	16	151 ± 19	448.6 ± 93.4
4. OVX+EB+P	8	137 ± 13	452.8 ± 45.4
5. OVX+P	7	168 ± 31	535.9 ± 67.5
6. Sham TX	8	232 ± 31	535.2 ± 63.5
7. TX	13	199 ± 24	556.7 ± 86.4
8. TX+T	14	218 ± 21	567.6 ± 63.4
9. TX+P	7	232 ± 19	572.2 ± 48.6
10. TX+EB+P	2	203 ± 11	509.5 ± 35.4

OVX: Ovariectomy.
TX: Orchiectomy.
EB: Estradiol Benzoate 20 μg/rat, twice a week.
T: Testosterone Propionate 2.5 mg/rat, twice a week.
P: Progesterone 10 mg/rat, twice a week.

Values are presented as means ± s.e.
P value $P < 0.001$ 2. vs 4.
$P < 0.01$ 2. vs 3.
$P < 0.05$ 1. vs 4., 3. vs 5., 4. vs 5.

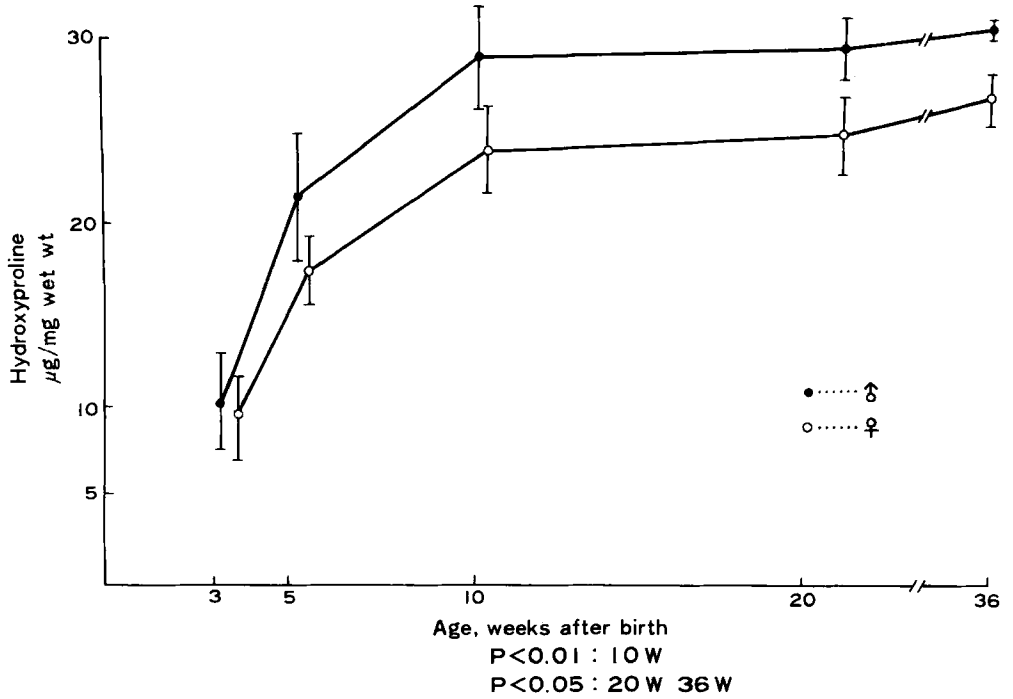

Figure 2. Influence of age and sex on collagen content of hip joint capsule in Wistar rat. Standard errors of means of hydroxyproline content are indicated by vertical lines.

Table 3. Influence of age and sex on collagen content of hip joint capsule in the Wistar rat.

Age (weeks)	Hydroxyproline (μg/mg wet wt.) Male	Female
3	10.22 ± 2.99 (6)	9.69 ± 2.39 (6)
5	20.87 ± 3.60 (7)	17.77 ± 1.91 (7)
10	28.63 ± 2.90 (5)	24.11 ± 2.32* (5)
20	29.73 ± 0.84 (5)	25.61 ± 1.04** (5)
36	30.47 ± 0.50 (3)	26.54 ± 1.62** (3)

Values are presented as means ± s.e.; the number of animals is given in parentheses.
* Statistically significantly different from males at $P < 0.01$.
** Statistically significantly different from males at $P < 0.05$.

Table 4. Influence of age and sex on collagen content of knee joint capsule in the Wistar rat.

Age (weeks)	Hydroxyproline (μg/mg wet wt.) Male	Female
3	4.26 ± 1.29 (6)	2.92 ± 1.04 (6)
5	4.37 ± 1.12 (7)	4.01 ± 0.31 (7)
10	9.19 ± 1.27 (5)	6.64 ± 1.39 (5)
20	9.24 ± 0.31 (5)	7.54 ± 0.35*(5)

Values are presented as means ± s.e.; the number of animals is given in parentheses.
* Statistically significantly different from males at $P < 0.05$.

with aging in both sexes. There was no significant sex difference in the fibril diameter, although it was larger in males than in females at any period examined (Table 1).

Following the administration of estrogen, or estrogen combined with progesterone, the fibril diameter was significantly decreased in the ovariectomized female rats. In the orchiectomized male rats, the fibril diameter was considerably increased by the administration of either progesterone or testosterone, whereas it was markedly decreased following the administration of estrogen combined with progesterone (Table 2, Figure 1).

Table 5. Effect of gonadectomy and hormone replacement on collagen content of hip joint capsule.

Exp. group	No. of animals	Body wt. (g)	Hydroxyproline μg/mg wet wt.
1. Sham OVX	12	156 ± 13	23.78 ± 2.16
2. OVX	28	180 ± 18	25.06 ± 2.83
3. OVX+EB	24	132 ± 11	22.97 ± 3.11
4. OVX+EB+P	14	133 ± 16	21.72 ± 3.15
5. OVX+P	13	176 ± 25	28.64 ± 3.66
6. OVX+T	14	182 ± 17	28.21 ± 2.13
7. Sham TX	8	232 ± 31	28.14 ± 3.49
8. TX	14	197 ± 24	25.85 ± 1.28
9. TX+T	14	218 ± 21	29.11 ± 4.66
10. TX+P	12	227 ± 19	25.35 ± 2.30
11. TX+EB+P	14	177 ± 16	22.12 ± 4.08
12. TX+EB	13	152 ± 19	24.09 ± 2.96

OVX: Ovariectomy.
TX: Orchiectomy.
EB: Estradiol Benzoate 20 μg/rat, twice a week.
T: Testosterone Propionate 2.5 mg/rat, twice a week.
P: Progesterone 10 mg/rat, twice a week.

Values are presented as means ± s.e.

P value		
	$P < 0.001$	1. vs 6., 1. vs 5., 2. vs 6., 3. vs 5., 3. vs 6., 4. vs 5., 4. vs 6., 9. vs 11.
	$P < 0.01$	2. vs 4., 8. vs 11., 7. vs 11., 9. vs 12.
	$P < 0.05$	2. vs 3., 7. vs 12., 8. vs 9., 9. vs 10., 10. vs 11., 7. vs 9., 7. vs 10.

Table 6. Effect of gonadectomy and hormone replacement on collagen content of knee joint capsule.

Exp. group	No. of animals	Body wt. (g)	Hydroxyproline μg/mg wet wt.
1. Sham OVX	6	149 ± 9	4.93 ± 0.90
2. OVX	25	179 ± 20	6.58 ± 1.13
3. OVX+EB	15	129 ± 10	5.30 ± 1.22
4. OVX+EB+P	14	133 ± 16	4.63 ± 1.02
5. OVX+P	13	176 ± 25	6.05 ± 1.44
6. OVX+T	14	182 ± 17	8.72 ± 2.34
7. Sham TX	4	218 ± 15	8.53 ± 1.37
8. TX	12	197 ± 19	6.77 ± 2.15
9. TX+T	9	214 ± 9	7.89 ± 0.83
10. TX+P	12	227 ± 19	5.77 ± 0.63
11. TX+EB+P	14	177 ± 16	3.73 ± 0.48
12. TX+EB	13	152 ± 19	4.18 ± 1.04

OVX: Ovariectomy.
TX: Orchiectomy.
EB: Estradiol Benzoate 20 μg/rat, twice a week.
T: Testosterone Propionate 2.5 mg/rat, twice a week.
P: Progesterone 10 mg/rat, twice a week.

Values are presented as means ± s.e.
P value $P < 0.001$ 1. vs 6., 1. vs 5., 2. vs 6., 3. vs 5., 3. vs 6., 4. vs 5., 4. vs 6., 9. vs 11.
$P < 0.01$ 2. vs 4., 8. vs 11., 7. vs 11., 9. vs 12.
$P < 0.05$ 2. vs 3., 7. vs 12., 8. vs 9., 9. vs 10., 10. vs 11., 7. vs 9., 7. vs 10.

Collagen content

As the age advanced, collagen content per unit wet weight of the hip joint capsule gradually increased up to the stage of sexual maturation but not after that, and it was significantly greater in males than in females at 10, 20 and 36 weeks of age (Table 3, Figure 2). In the knee joint capsule, there were similar tendencies to those observed in the hip joint capsule, although collagen content per unit wet weight was less in the former than in the latter (Table 4).

The collagen content was significantly increased by ovariectomy and decreased by the administration of estrogen, or estrogen combined with progesterone, while it was significantly increased by the administration of progesterone or testosterone alone in the ovariectomized rats (Table 5).

The collagen content of the knee joint capsule was also significantly increased by ovariectomy with or without administration of testosterone, but it was significantly decreased by the administration of estrogen, or estrogen combined with progesterone, in the ovariectomized female rats (Table 6).

DISCUSSION

There have been conflicting views concerning the effect of sex hormones on connective tissue metabolism. The general consensus, however, seems to be that collagen accumulation is greater in males than in females (Fischer 1973). This opinion is well documented by the present study, i.e. collagen content per unit wet weight of the joint capsule is significantly greater in males than in females after the stage of sexual maturation. The influence of sex hormones on collagen content is also well demonstrated by the fact that the collagen content is approx-

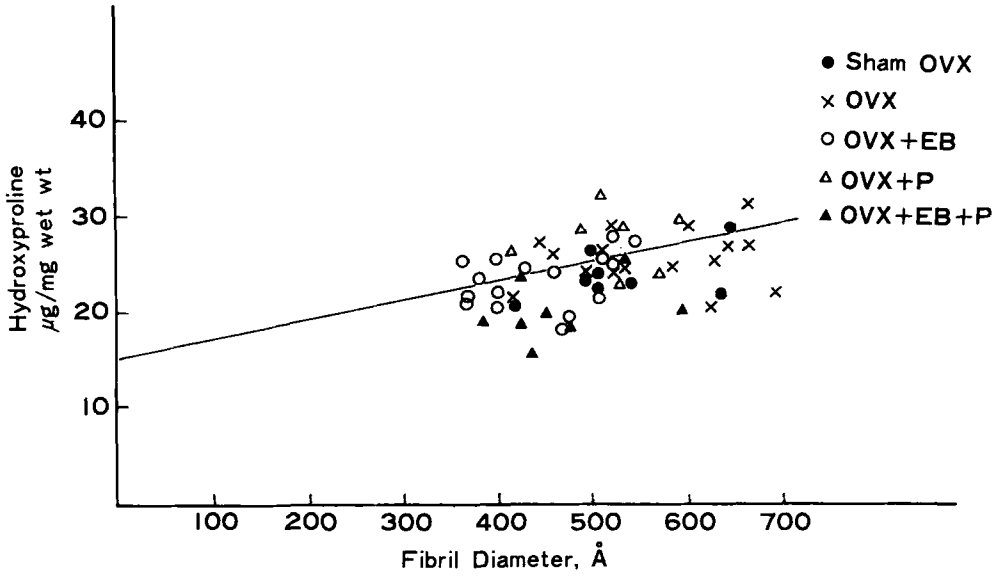

Figure 3. Correlation between collagen content and fibril diameter of hip joint capsule in female Wistar rat.

imated in both sexes after gonadectomy.

It has been said that estrogen accelerates collagen synthesis in the rat uterus (Kao et al. 1964) and in rat bone (Orimo et al. 1971), whereas it inhibits collagen synthesis in the rat tail tendon (Fischer 1973). It is made clear by the present study that the collagen content of both hip and knee joint capsule is significantly decreased by estrogen or estrogen combined with progesterone. It is not known whether the decrease is derived from the inhibition of collagen synthesis or the stimulation of collagen degradation.

The results of the present experiment indicate that there is a close interrelationship between collagen content and fibril diameter in the joint capsule. A positive correlation between them is found statistically in females (Figure 3). However, factors that control collagen fibril diameter have not been elucidated. It has been stated that there might be an inverse relationship between collagen fibril diameter and collagen hexose content, and that the location of the hexose on specific hydroxylysine residues might hinder the formation of intermolecular cross-links (Grant et al. 1969, Morgan et al. 1970). Estrogen, or estrogen combined with progesterone, is supposed to influence the glycosylation of these residues, but this remains to be clarified.

Biological and morphological changes of collagen fibers such as those mentioned above may result in changes in the mechanical properties of the connective tissue. Therefore, it is probable that an imbalance of estrogen metabolism is related to relaxation of the hip joint capsule, although the relaxation may not necessarily be the primary etiological factor in congenital dislocation of the hip.

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