# The role of sex hormones in the kinetics of chondrocytes in the growth plate

## A STUDY IN THE RABBIT

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**Sex hormones play important roles in the regulation of the proliferation, maturation and death of chondrocytes in the epiphyseal growth plate. We have investigated the effects of male castration on the cell kinetics of chondrocytes as defined by the numbers of proliferating and dying cells. The growth plates of normal rabbits and animals castrated at eight weeks of age were obtained at 10, 15, 20 and 25 weeks of age.**

**Our study suggested that castration led to an increase in apoptosis and a decrease in the proliferation of chondrocytes in the growth plate. In addition, the number of chondrocytes in the castrated rabbits was less than that of normal animals of the same age.**

The growth plate of a long bone is divided morphologically and functionally into three zones: resting, proliferating and hypertrophic. Resting chondrocytes located on the epiphyseal side of the growth plate are stimulated to proliferate and then to hypertrophy through several maturation stages.[1-3] It is generally accepted that the terminal chondrocytes undergo apoptosis or programmed cell death.[4-8] During these processes, several hormones such as sex steroids and some cytokines, for example insulin-like growth factor-1, play important roles.

Androgens and oestrogens are recognised as male and female hormones, respectively, but both are present in each sex.[9] They induce secondary sexual characteristics and give rise to the pubertal growth spurt.[10-13] The androgens include several hormones such as testosterone, a major part of androgen, 1-dihydrotestosterone, 5-dihydrotestosterone and androsterone. About 95% of testosterone is produced by the Leydig cells in the testis and the rest from the adrenal gland in both sexes.[14] The hormone stimulates the activity of accessory male sexual organs and promotes the development of male sexual characteristics, the formation of sperm and the anabolic action in musculoskeletal development.[15-17]

Castration models have been used for many years to study the effects of male hormones on the growth of long bones.[17-23] The effects of castration on growth and on the chondrocytes in the growth plate remain unclarified. We have therefore performed an immunohistological study *in vivo* using markers for apoptosis and for proliferation to investigate the kinetics of chondrocytes in the growth plate in castrated male rabbits.

## Materials and Methods

The experiment was carried out according to the guidelines of the Ethics Committee of Tohoku University. Thirty-two male Japanese white rabbits were divided into eight groups of four. The normal rabbits were killed by intravenous injection of pentobarbital sodium at 10, 15, 20 and 25 weeks of age and were designated as the 10W, 15W, 20W and 25W groups. Sixteen rabbits were castrated at eight weeks and killed at 10, 15, 20 and 25 weeks. They were described as the 8-10W, 8-15W, 8-20W and 8-25W groups. The level of serum testosterone in both the normal and castrated rabbits was measured at the time of castration and when they were killed using a radioimmunoassay solid phase procedure. The right leg of each rabbit was dismembered and the length of the femur was measured using soft radiography (low power > 30 kv).

**Tissue preparation and morphological observation.** The epiphyseal growth plate, together with the metaphyseal and epiphyseal bone, was taken from the centre of the femoral head of each rabbit. The growth plates were sectioned coronally into blocks 5 mm thick. These were fixed in 10% formalin for three days, decalcified by ethylenediaminetetraacetic acid (EDTA) at 4°C for ten days, embedded in paraffin and cut into slices 2.5 μm thick.[24-26]

After deparaffinisation and rehydration, some sections from each specimen were stained

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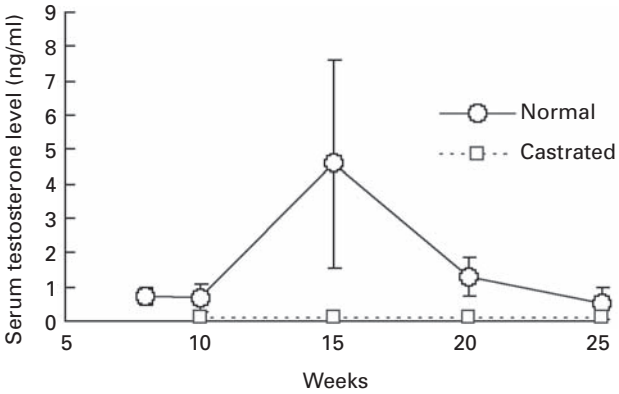


Fig. 1

The serum level of testosterone in the normal and castrated rabbits.

**Table I.** The mean (SD; cm) length of the rabbit femora according to age in both groups

| | Age (wks) | | | |
|---|---|---|---|---|
| **Group** | **10** | **15** | **20** | **25** |
| Normal | 8.2 (0.2) | 9.3 (0.2) | 9.8 (0.4) | 10.1 (0.7) |
| Castrated | 8.1 (0.6) | 9.5 (0.5) | 10.2 (0.2) | 10.4 (0.4) |

with haematoxylin and eosin (HE). These were used to determine the morphological changes in the growth plate with age. The height of the growth plate was measured microscopically at six points which were located at regular intervals within each growth plate. Immunostaining for osteonectin (ONT) was performed using the labelled streptavidin-biotin (LSAB) method with a reaction time of 60 minutes for anti-ONT antibody (Takara Bio Inc, Otsu, Japan) at a dilution of 1:100 in order to detect the hypertrophic chondrocytes which had strongly-stained cytoplasm.[25,27] Both HE and immunostained sections were used to determine the division of the growth plates into the three zones. The cell numbers were counted three times in five fields selected randomly at a magnification of x200 on the HE sections.[24-26]

**Immunohistochemistry.** For *in situ* visualisation of apoptotic cells we performed immunostaining for caspase-3, which is activated downstream of initiator caspases and is essential for the regulation of apoptosis in the chondrocytes of the growth plate.[28,29] The sections were stained using the avidin-biotinylated peroxidase complex (ABC) method with a reaction time of 60 minutes for anti-caspase-3 antibody (Biotech Inc., Santa Cruz, California) at a dilution of 1:50. They were counterstained using haematoxylin. Proliferating chondrocytes were detected by immunostaining for proliferating cell nuclear antigen (PCNA), an auxillary protein of DNA polymerase.[30] The LSAB method with a reaction time of 60 minutes for anti-PCNA antibody (Dako, Cytomation, Glostrup, Denmark) at a dilution of 1:50 was used. Counterstaining was carried out using Methyl Green. In both caspase-3 positive and PCNA immunostaining, negative controls were incubated with mouse serum before processing and only background staining was found.

For each antibody and each rabbit block, immunocytochemistry for caspase-3, PCNA and ONT was performed on several sections until at least two showed the same immunostaining pattern.

**Quantification of caspase-3 and PCNA expression.** Chondrocytes with diffusely stained cytoplasm or nuclei after immunostaining for caspase-3 were regarded as positive cells.[31] Those with diffusely stained nuclei for PCNA were also positive. The caspase-3-positive and the PCNA-positive ratios were calculated in the whole growth plate and in the three zones.[24-26] The ratios in the castrated groups were compared with those of the normal groups of similar age.

**Statistical analysis.** This was performed to determine the significance of the differences in the caspase-3 and the PCNA-positive ratios between the normal and castrated groups at each age. We used an unpaired Student's *t*-test between the paired groups at 10, 15, 20 and 25 weeks. The level of significance was set at $p < 0.05$. All statistical analyses were performed with commercially available software (Statview; Abacus Concepts, Berkeley, California).

## Results

In the normal rabbits, less than 1 ng/ml of serum testosterone was detected at 8 and 10 weeks. The highest level, 4.7 ng/ml, was recorded at 15 weeks after which it decreased again at 20 and 25 weeks (Fig. 1). In the castrated rabbits, the serum testosterone was below the detection limit of 0.1 ng/ml (Fig. 1). There was no significant difference in the femoral length of the normal and castrated groups at the same age (Table I).

In the normal rabbits, the proliferating and upper hypertrophic chondrocytes maintained obvious columnar structures until 20 weeks of age with a decreasing number of chondrocytes, particularly in the proliferating zone (Figs 2a to 2c, top). In the 25W group, one of the four rabbits showed closure of the growth plate and the other three disarrangement of the columnar structure and narrowing of all zones. Some areas had a wide growth plate with many hypertrophic chondrocytes and a few proliferating cells (Fig. 2d) and others had a few chondrocytes within an irregular narrowed cartilage matrix. In each age group, the cells with large round to oval cytoplasm and those stained positively with ONT were considered to be hypertrophic chondrocytes (Figs 2e and 2f, top).

In the castrated rabbits in the 8-10W group, the columnar structure and the morphology of the chondrocytes in the proliferating zone were almost similar to those of the 10W group but between the proliferating and hypertrophic zones, these colums became disarranged (Fig. 2a, bottom). In the 8-15W group, the flattened proliferating cells were

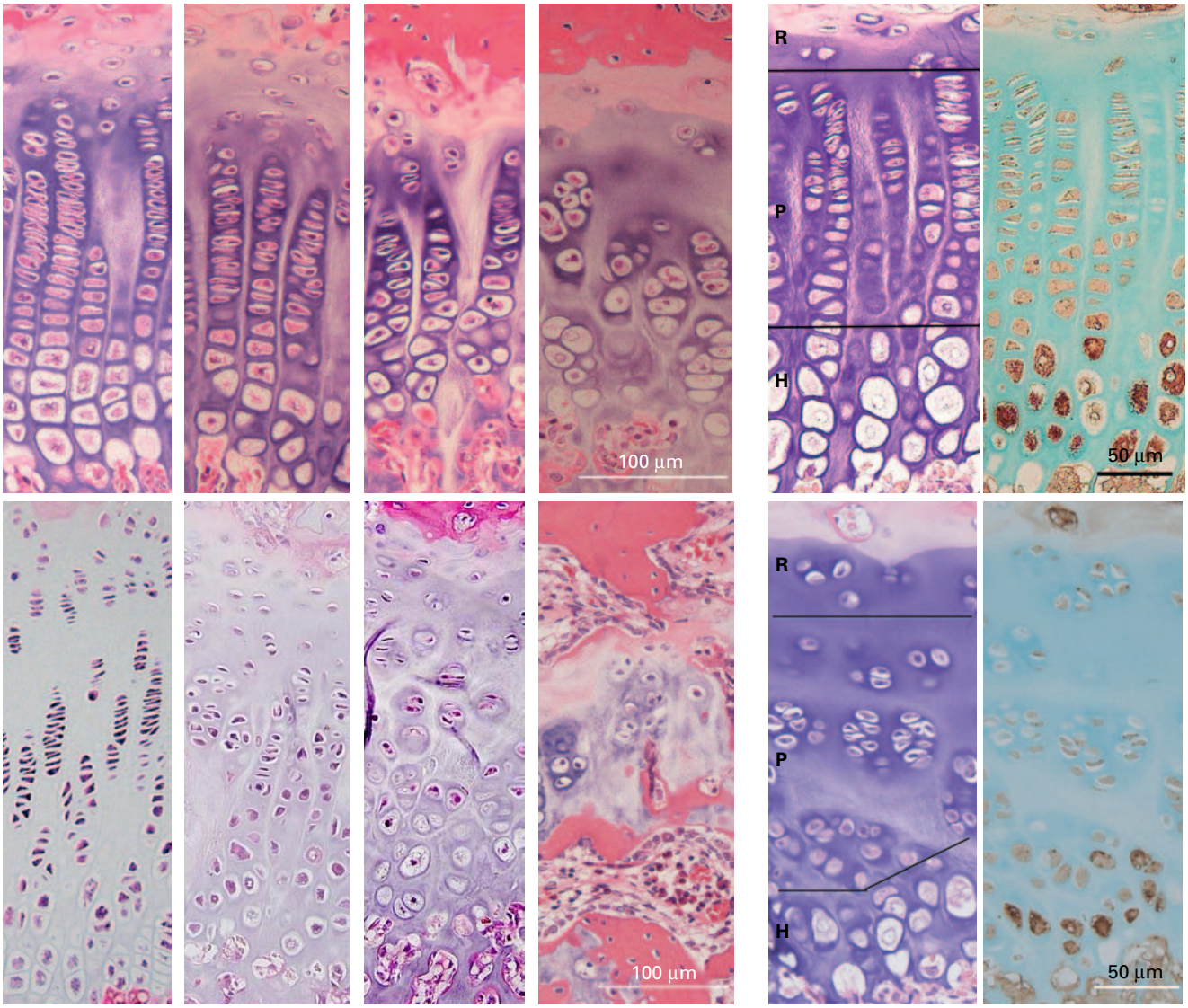


Fig. 2a Fig. 2b Fig. 2c Fig. 2d Fig. 2e Fig. 2f

The growth plate in the normal (top) and castrated (bottom) rabbits. Figures 2a to 2d – Photomicrographs at a) 10, b) 15, c) 20 and d) 25 weeks of age (haematoxylin & eosin (HE) x120). Figures 2e and 2f – Photomicrographs at 15 weeks using e) HE and f) immunostaining for osteonectin (x120). The growth plate is divided into resting (R), proliferating (P) and hypertrophic zones (H).

obviously decreased in number and the proliferating zone was narrowed (Fig. 2b, bottom). In this group, slightly flattened chondrocytes, located in the upper part of the hypertrophic zone, stained negatively for ONT (Figs. 2e and f, bottom) indicating that they were proliferating cells. In the 8-20W group, the columnar structure was irregular and the cellularity was reduced, particularly in the proliferating zone (Fig. 2c, bottom). In the 8-25W group, the growth plate was closed in one of the four rabbits. In the other rabbits, a few hypertrophic chondrocytes were still detected (Fig. 2d, bottom).

The height of the growth plate of the normal rabbits in the 10W, 15W, 20W and 25W groups measured 432 (SD 32), 357 (SD 20), 324 (SD 42) and 206 µm (SD 149), respectively. That of the castrated animals in the 8-10W, 8-15W, 8-20W and 8-25W groups was 423 (SD 70), 297 (SD 9), 217 (SD 69) and 70 µm (SD 68), respectively. The differences between the 15W and 8-15W groups and between the 20W and 8-20W groups were statistically significant. Those at 25 weeks were not significant because of the large variation among animals. Overall, the height of the growth plate was much narrower after castration than that in the normal rabbits, particularly at 20W and 25W. The timing of the closure of the growth plate, however, did not indicate much difference between the normal and castrated groups.

The cell numbers of the whole growth plate and the three divided zones are shown in Figure 3. The number of cells decreased in both the normal and castrated groups. At a

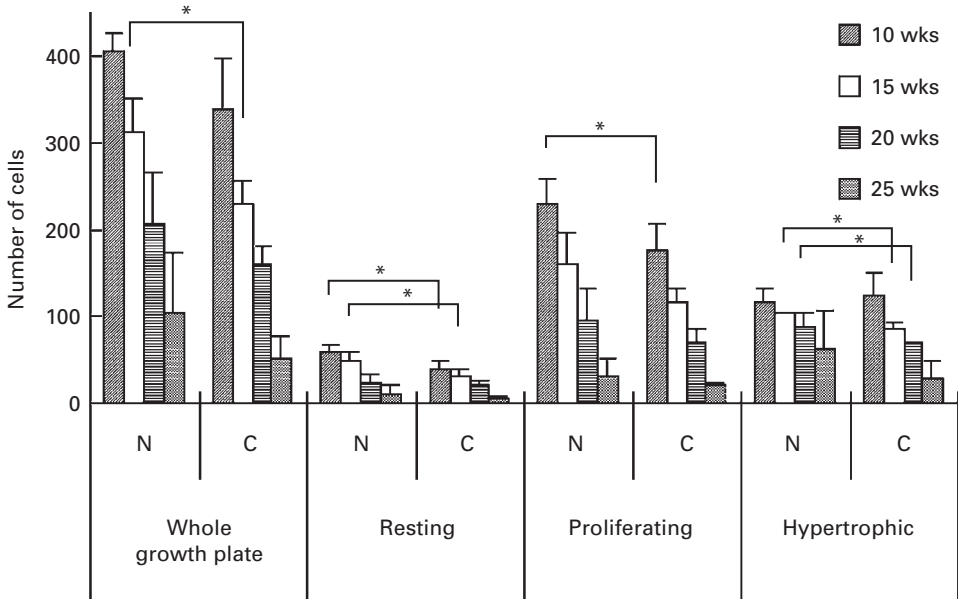


Fig. 3

Histogram showing the mean (SD) number of chondrocytes in the whole growth plate and individual zones over the various time periods in the normal (N) and castrated (C) rabbits.

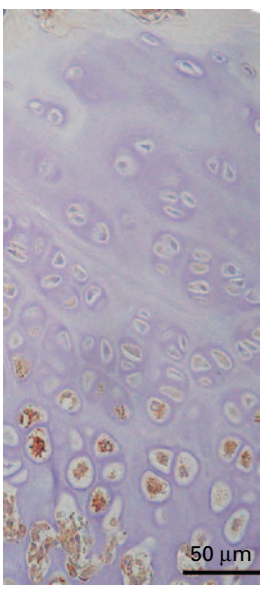


Fig. 4

Photomicrograph showing immunostaining for caspase-3 in a growth plate of a castrated 20-week-old rabbit (x200).

given age, higher cellularity was always observed in the normal than in the castrated group.

Caspase-3-positive chondrocytes were found in every harvested growth plate of both the normal and castrated rabbits, mainly in the hypertrophic zone (Fig. 4). The caspase-3-positive ratios of the normal rabbits in the whole growth plate at 10, 15, 20 and 25 weeks was 8.7 (SD 1.6), 9.1 (SD 2.5), 11.8 (SD 1.8) and 15.0% (SD 5.4), respectively. The positive ratio in the castrated rabbits of the 8-10W, 8-15W, 8-20W and 8-25W groups was 8.1 (SD 0.7), 10.2 (SD 1.8), 13.3 (SD 3.6) and 19.5% (SD 3.3), respectively. These ratios were higher than those in the corresponding normal groups. No statistically significant difference was found between the normal and castrated groups over the entire growth plate and in the three divided zones (Fig. 5). In the hypertrophic zone, where most caspase-3-positive cells were detected, the ratio in the normal and castrated groups increased with age and the latter ratio was higher than the former. Conversely, in the resting and proliferating zones, the positive ratio was extremely small and there was little change with age and little difference between the two groups of the same age.

PCNA-positive chondrocytes were found in every harvested growth plate of both the normal and castrated rabbits and in all three individual zones. Most of the positive chondrocytes were found in the proliferating zone and in the upper hypertrophic zone (Fig. 6). The PCNA-positive ratio of the normal rabbits in the whole growth plate at 10, 15, 20 and 25 weeks was 14.5 (SD 2.5), 12.5 (SD 2.9), 7.8 (SD 1.2) and 5.1% (SD 0.3), respectively. That in the 8-10W, 8-15W, 8-20W and 8-25W groups was 10.8 (SD 1.0), 5.8 (SD 1.7), 4.5 (SD 0.4) and 4.0% (SD 1.2), respectively. The ratio decreased with age in both the normal and castrated groups with that in the castrated rabbits being lower than the ratio in the corresponding normal rabbits. There were significant differences between the 10W and 8-10W groups, the 15W and 8-15W groups, and the 20W and 8-

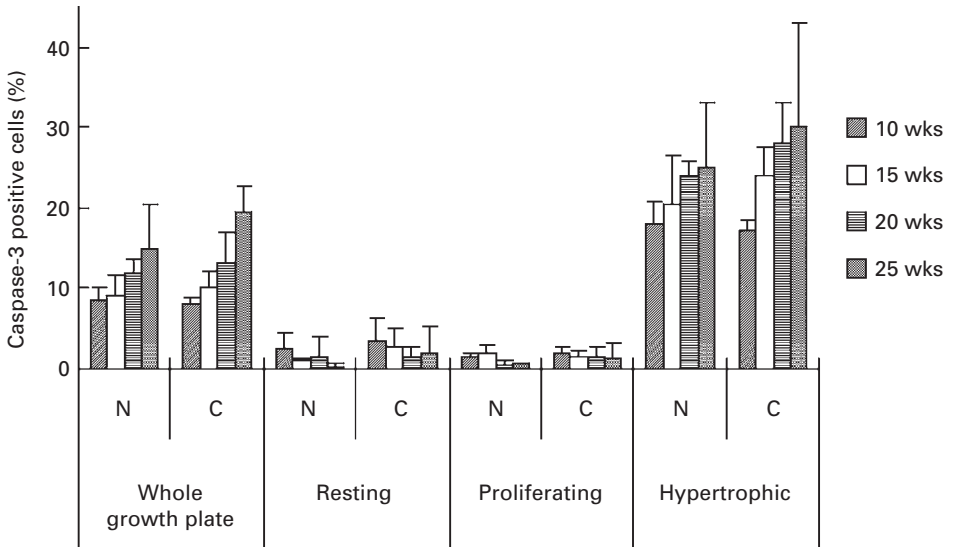


Fig. 5

Histogram showing the mean (SD) caspase-3-positive ratios in the whole growth plate and three divided zones in the normal (N) and castrated (C) rabbits. Each bar represents the mean (SD) (x200). The ratios increase with growth in the whole growth plate and the hypertrophic zone in both rabbits.

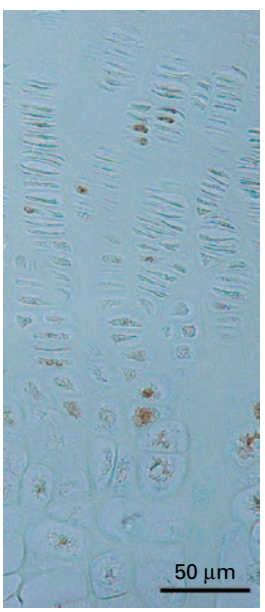


Fig. 6

Photomicrograph showing immunostaining for PCNA in a growth plate of a 10-week-old rabbit. Positive chondrocytes with diffusely-stained nuclei were found mainly in the proliferating and upper hypertrophic zones (x200).

20W groups (Fig. 7). In addition, in the three divided zones the positive ratio tended to decrease with age and the castrated groups showed a lower positive ratio than the corresponding normal groups. However, a significant difference was found only between the proliferating zones of the 15W and 8-15W groups (Fig. 7).

## Discussion

The increase in the level of testosterone initiates puberty and promotes pubertal changes including epiphyseal maturation.[9,32-34] In our study, the serum level of testosterone remained low until ten weeks but was maximal at 15 weeks in the normal Japanese white rabbits, which suggested that the prepubertal phase lasted at least until ten weeks of age. Therefore, it was thought reasonable to castrate the rabbits at eight weeks in order to study the effects of testosterone since they had not, by then, been exposed to male hormones.

Testosterone acts through at least three pathways. First, it converts to oestrogen which accelerates growth and epiphyseal maturation.[9] Secondly, it increases production of growth hormone and insulin-like growth factor (IGF-1), which stimulate proliferation of the chondrocytes in the growth plate.[20,33,34] Finally, it stimulates the proliferation of chondrocytes by local upregulation of IGF-1 receptor gene expression.[20] Testosterone accelerates proliferation of the chondrocytes of the growth plate *in vivo*, although *in vitro*, it inhibits proliferation,[9,20,33-36] which suggests that testosterone has little direct effect on the proliferation of chondrocytes in the growth plate *in vivo*.

The effects of testosterone on the death of chondrocytes are less understood. Okada[37] reported that it increased apoptosis in the resting and hypertrophic zones *in vitro* and that oestrogen also stimulated apoptosis of chondrocytes *in vivo* and *in vitro*. IGF-1 has been detected in terminal hypertrophic chondrocytes suggesting that it has a function of local control of the initiation of terminal cells into apop-

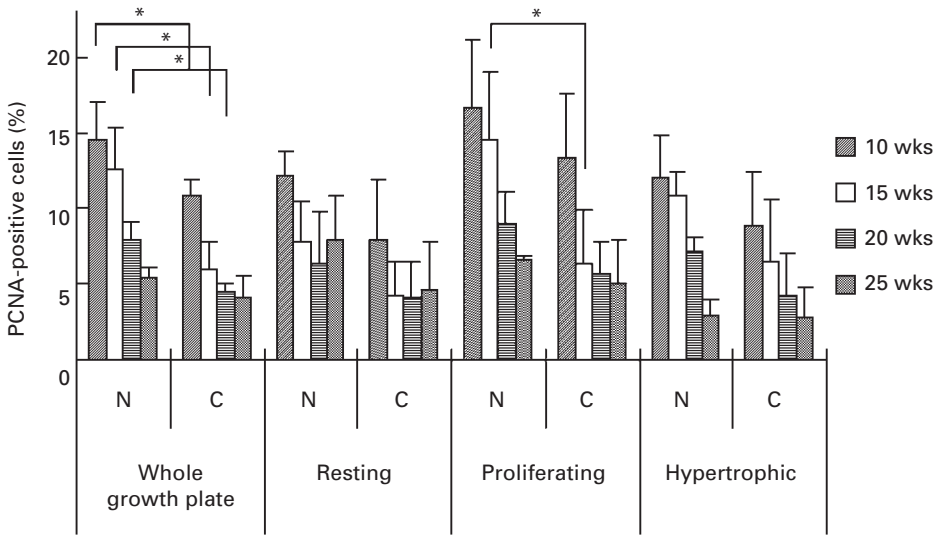


Fig. 7

Histogram showing the mean (SD) PCNA-positive ratios in the whole growth plate and the three divided zones. The ratios decrease with growth in the normal (N) and castrated (C) rabbits.

tosis.[38] On the other hand, IGF-1 showed anti-apoptotic effects on intervertebral disc cells *in vitro*.[39] Growth hormone inhibited apoptosis in osteoblast-like cells and myoblasts.[40,41]

The effects of castration on the growth of long bones remains controversial. Some studies have reported an increased length of long bones and delayed closure of the growth plate in castrated animals while others described a decrease in the length.[15,17,21,22] In addition, the effects of castration on chondrocytes in the growth plate remain unclarified. An increased number of proliferating chondrocytes and a delay in hypertrophy have been reported in castrated animals,[15,17] while a decreased ability to synthesise DNA after gonadectomy has recently been described.[23] These diverse results may be caused by a difference in the animal model that was used. In addition, the testosterone pathway which is dominant may depend on the type and the age of the animals.[15,17,21-23]

The maturation involving mineralisation and ossification of the epiphyseal growth plate is mainly controlled by oestrogen.[9,21,22] In our study, the castrated and normal rabbits had almost the same timing of closure of the growth plate although the former showed a tendency for a decline in the height and cellularity of the growth plates. This may be because the oestrogen produced from testosterone would have been low in the castrated rabbits.

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