INTRODUCTION:
Longitudinal bone growth is regulated by chondrocytes of the epiphyseal growth plate, in which cell proliferation, differentiation and programmed cell death (apoptosis) occur consecutively. Growth hormone (GH), insulin-like growth factor-1 (IGF-1), thyroid hormones and glucocorticoids play major roles as systemic regulators in these long bone growth processes. In addition, sex steroids, such as estrogen and androgen, are important regulators, particularly during puberty.

Estrogen is a major sex hormone and mainly secreted by the ovary in female and also derived from testis and from extragonadal aromatization of testosterone and androstenedione in male. The androgens include several hormones including testosterone that is a major part of androgen. About 95% of testosterone is produced by the Leydig cells in the testis and the rest from the adrenal gland in both sexes. Many studies have been reported on the roles of both sex hormones to the regulation of long bone growth, however, some of them have remained unclarified to date including those for cell kinetics in the growth plate chondrocytes. The purpose of this study was to clarify the effect of the deficiency of sex hormones on growth plate chondrocytes. We have therefore performed an immunohistological study in vivo using markers for apoptosis and for cell proliferation in the growth plate chondrocytes in gonadectomized rabbits.

MATERIALS AND METHODS:
Animals: Thirty-two each gender of Japanese white rabbits were divided into sixteen groups of four. Sixteen each sex were sham-operated used as the control groups; only skin incision and subsequent suture were performed at 8 weeks. Another sixteen rabbits of each gender were gonadectomized at 8 weeks. All animals were euthanized at 10, 15, 20 and 25 weeks of age by intravenous injection of overdose pentobarbital sodium. The rabbits in the control group designated as the 10W, 15W, 20W and 25W groups and those in the gonadectomized group as the 8-10W, 8-15W, 8-20W and 8-25W groups, respectively depending on the age of euthanasia.

Tissue preparation and morphological observation: The epiphyseal growth plate was taken from the center of the femoral head of each rabbit. The sections stained with haematoxylin and eosin (HE) were first used to investigate and to measure the morphological changes of growth plate. The cell numbers were counted three times in five fields randomly selected at a magnification ×200 in the whole growth plate and the three divided zones: resting, proliferating and hypertrophic.

Immunohistochemistry: Apoptotic chondrocytes were visualized in situ by immunostaining for caspase-3, while cell proliferation was detected by immunostaining for proliferating cell nuclear antigen (PCNA). The avidin-biotinylated peroxidase complex (ABC) method was used for the immunostaining of caspase-3 with anti-caspase-3 antibody (Biotech Inc., Santa Cruz, California) and the labeled streptavidin-biotin (LSAB) method for PCNA with anti-PCNA antibody (Dako, Cytomation, Glostrup, Denmark).

Quantification of caspase-3 and PCNA expression: Chondrocytes with diffusely stained cytoplasm or nuclei by anti-caspase-3 or stained nuclei by anti-PCNA were regarded as positive cells. The caspase-3-positive and PCNA-positive ratios were calculated in the whole growth plate and in three divided zones. The ratios of the gonadectomized rabbits were compared with those of the control ones at similar age.

Statistical analysis: Differences between groups were examined using an unpaired Student’s t-test. Results were significant at p<0.05.

RESULTS:
The serum level of estrogen and testosterone was under the limitation of detection after 10-week of age. The height of the growth plate in the castrated rabbits was much narrower than that in the control rabbits while the ovariectomized groups tended to have higher than the control ones.

The caspase-3 and PCNA positive chondrocytes were found in all divided zones of all rabbit growth plates. Caspase-3-positive chondrocytes are shown in Figure 1. The positive ratio tended to increase with age and the gonadectomized groups showed a higher positive ratio than the corresponding control groups. PCNA-positive chondrocytes are shown in Figure 2. The positive ratio tended to decrease with age and the gonadectomized rabbits had statistically significant lower positive rates than the control ones. The number of chondrocytes decreased with age in the both control and gonadectomized groups and the latter showed fewer numbers than the corresponding control groups.

DISCUSSION:
The effects of both sex hormones are mediated directly or through growth hormone and insulin-like growth factor-1 (GH-IGF-1) axis. Direct effect of estrogen depends on the receptors it binds: estrogen receptor (ER)-alpha accelerates longitudinal bone growth while ER-beta represses it. Testosterone mainly converts to estrogen by aromatase and also directly accelerates chondrocyte proliferation in vivo. The present study indicated that the deficiency of either estrogen or testosterone on the growth plate chondrocytes more strongly acted in the decreased of proliferation ability than in the increase of chondrocyte apoptosis, which led the reduction of the number of chondrocytes. In rabbits, the pathway through ER-alpha or GH-IGF-1 axis might be dominant and testosterone might have little effect directly in the cell kinetics of growth plate chondrocytes.