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BLASTEMA VOLUME AND NUCLEAR SIZE IN THE REGENERATING HIND LIMBS IN LARVAE OF *XENOPUS LAEVIS* AND *BUFO REGULARIS* AFTER TRANSECTION AT THE ANKLE LEVEL

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Key words: Xenopus, Bufo, Blastema, nuclear size.

ABSTRACT

After amputation at the ankle level of hind limbs in larval *Xenopus laevis* and *Bufo regularis,* an increase in the nuclear volume of blastema and stump tissue cells was observed. The cells in the distal region of blastema appeared to have the largest nuclei throughout the whole regenerating limb. In the stump, the increase in the nuclear volume was mostly confined to the distal region close to the amputation site, and a proximodistal gradient was pronouced. This might explain that amputation increased the metabolic activities of the stump tissue cells. There was a general indication that the nuclear volume value was higher in *Xenopus* than in *Bufo.*

Measurments of blastema and stump volumes revealed that although the blastema size at the late larval stage 57 was larger than that of the early stage 53; yet the value of blastema volume relative to stump volume was much higher at the early stage. This value was significantly larger in *Xenopus* than in *Bufo.* The nuclear volume was positively correlated with blastema volume at stage 53, whereas at stage 57, this correlation was negative. This may indicate that the mesenchymal blastema size seems to be a reflexion to the nuclear size, than to the intercellular substance.

INTRODUCTION

During transformation of dedifferentiated tissue cells into blastema cells their nuclei become larger and the cells become histologically approaching the mesechyme type in appearance (Hay and Fischman, 1961). The observations of Anton and Darda (1969) on protein synthesis and volume ratio in *Triturus,* revealed that the increase of protein synthesis rate in an activated cell, with enlarged nucleus, in comparison to a normal one, may be due to the activation of protein synthesis in the nucleus. An increase in nuclear volume as well as in protein synthesis was observed in epidermal tissue of regenerating *Triturus* forelimbs (Anton and Elsen, 1979; Anton and Bourauel, 1982).

The factors influencing the growth regeneration blastema were earlier investigated (Singer and Craven, 1948; Tweedle, 1971; Pritchett and Dent, 1972; Schauble, 1972; Iten and Bryant, 1973; Smith *et al.,* 1974; Conneley, 1977). In most of these studies, morphological and histological changes of blastema were expressed as a function of time after amputation. There was a considerable variation in developmental morphology of limb blastemas with post-amputational age (Iten and Bryant, 1973).

The present study was carried out to investigate statistically the changes in the nuclear volume, blastema and stump volumes during early stages of regeneration in both *Xenopus* and *Bufo.*

MATERIAL AND METHODS

Larvae of *Xenopus laevis* and *Bufo regularis* were reared in the laboratory at a temperature about 25 ± 1°C and staged according to the Normal Tables of Neiuwkoop and Faber (1965) for *Xenopus laevis* and of Sedra and Michael (1961) for *Bufo regularis.* The early and late larval stages numbers 53 and 57 were used in the study.

After narcotizing the animals with MS 222 (conc.,1:4000), the left hind limb was transected at the middle level of ankle segment. Limbs were fixed after 1,4 and 7 days. Serial longitudinal sections (5μ thick) were prepared and stained with nuclear fast red-Aluminium sulfate.

The areas of the nulei of blastema cells as well as of cells of some stump tissues were determined using Graphics Tablet (Apple Computer, Inc.) and the data were analysed by the aid of computer programs. Generally, the nuclear volume is proportional with the nuclear areas, and therefore the increase in the mean of nuclear area corresponds to the increase in the mean of nuclear volume. Areas measurements were carried out in relative estimation fields along the proximodistal axis of the limb.

Measurements of blastema and stump volumes

Serial longitudinal sections were stained with nuclear fast red and counterstained with modified Azan (aniline blue-orange G). The clear cut-ends of the basement membrane as well as stump corium could be used as natural postoperation markers to define the amputation plane and the boundaries between the blastema or the regenerate and the stump itself. The pigmentation differences between the stump and the regenerate could also be used as marker.

In every third section, the outline of blastema (without covering epidermis) as well as of the stump (with epidermis) was drawn with the aid of a camera lucida at a magnification of X 80. The area of each drawing was determined using the Graphics Tablet, and the total volume of blastema and that of stump were calculated by the aid of adapted Computer programs. These measurements were carried out on 5 animals for each stage, fixed 7 days after amputation. Thus, for comparison of blastema or stump volume between *Xenopus* and *Bufo*, the post-amputational age and the developmental stage were constant.

RESULTS

Nuclear volume

Blastema cells

At stage 53, early mesenchymal blastema was established by the fourth day post-amputation. It increased in size and its cells increased in number due to the active cell division. However, by stage 57, a fibrocyte-like blastema was formed by the seventh post-amputation day. In the regenerate in both stages: 53 and 57, in *Xenopus* and *Bufo*, the nuclear volume of blastema cells (Fig. 1) increased so much, reaching by the seventh day post-amputation more than the double the value on the first day (cell debris and dedifferentiated cells). The distal blastema region was occupied by the most largest cells in the regenerating limb, while the proximal region showed smaller cells, and a proximodistal gradient in nuclear volume was pronounced in boh species.

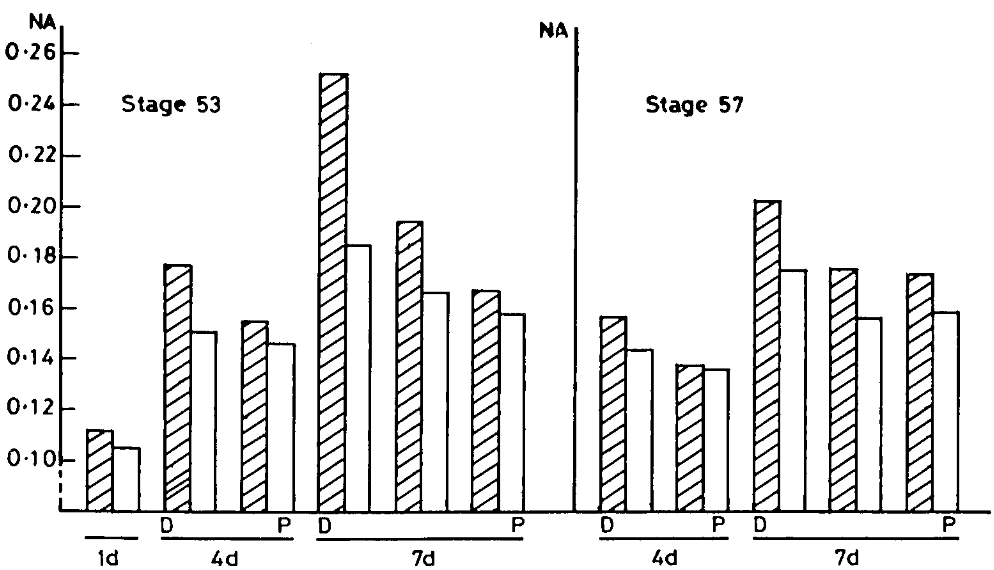

Fig. 1: Histogram illustrating the mean area of nuclear sections (NA) of blastema cells at stage 53 (left) and stage 57 (right) along the proximodistal axis of the regenerates fixed 1,4, and 7 days after ampulation, in *Xenopus* (diagonally stripped bars) and in *Bufo* (empty bars). Abscissa represents relative estimation fields from distal (D) to proximal (P) regions. P, regions at blastemal base.

Stump tissues

Stage 53

The nuclear volume of cartilage cells along the proximodistal axis of the control limb was nearly uniform (Fig. 2 & 3). After amputation that volume increased showing its peak distally by the seventh day, which was even higher than in the control limb. In the stump mesenchyme cells, the nuclear volume seemed to be uniform from proximal to distal regions along the control limb and it increased during development. A proximodistal gradient in the nuclear volume was revealed by the fourth and seventh days post-amputation. The maximal value (20.42) which was evident by the seventh day in the distal region near the amputation plane, was much higher than in the control limb (16.20). In the corium, the volume seemed to be nearly uninfluenced directly by amputation, it progressively increased showing higher value distally than proximally.

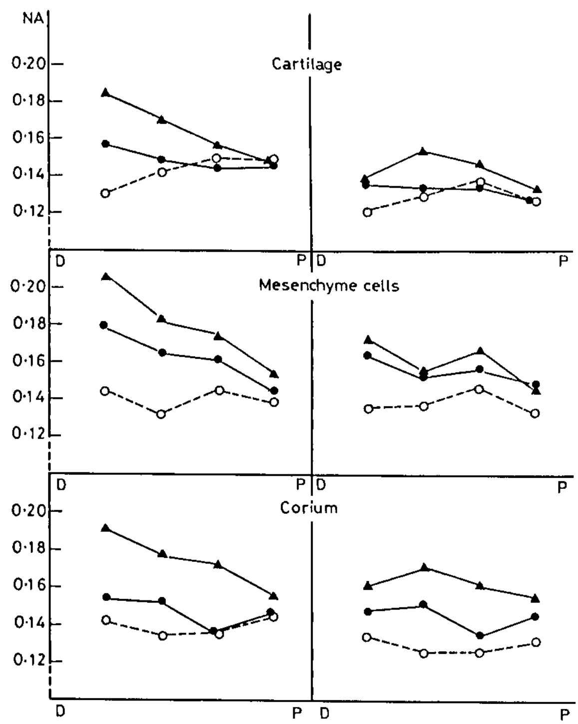

Fig. 2: Mean area of nuclear sections (NA) of cartilage, mesenchyme and corium cells at stage 53 of *Xenopus*, along the proximodistal axis of experimental limbs (left), fixed 1,4 and 7 days after amputation, as well as of control limbs (right). Abscissa represent relative estimation fields from distal (D) to proximal (P) regions. P regions at base of stump. ---o---, 1 day ; —●—, 4 days; —▲— 7 days.

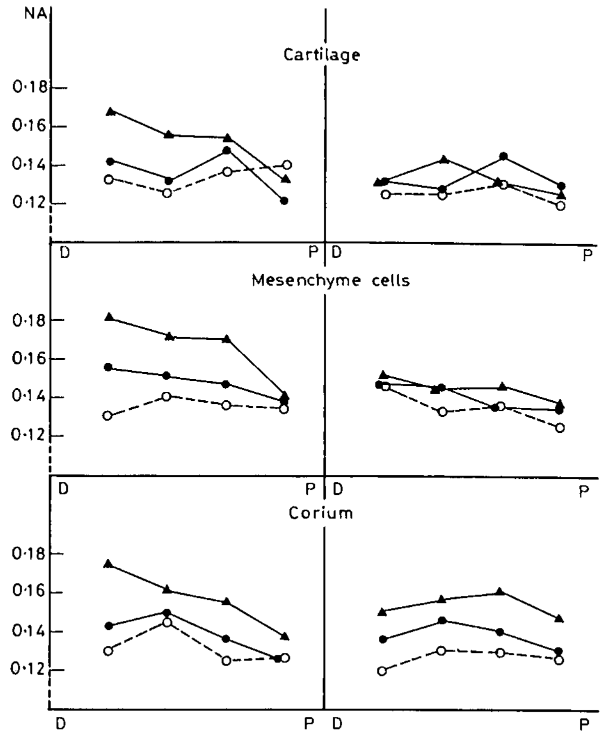

Fig. 3: Mean area of nuclear sections (NA) of cartilage, mesenchyme and corium cells at stage 53 of *Bufo*, along the proximodistal axis of experimental limbs (left), fixed 1,4 and 7 days after amputation, as well as of control limbs (right). Other abbreviations as in fig. 2.

Stage 57

The mature chondrocytes of experimental limbs displayed, by the seventh day post-amputation higher values in nuclear volume distally than proximally (Fig. 4 & 5); these values were compartively higher than in the control limbs, in which there was a slight increase in the nuclear volume during development. In the connective tissue cells, the nuclear volume was slightly changed from the control limb and up till the fourth day post-amputation. Thereafter by the seventh day, it increased distally so that a proximodistal gradient was distinguished. In the corium, there was nearly no distinct influence in the nuclear volume by the first day. However, it increased continuously by the fourth and seventh days, showing its maximal values distally.

It was generally observed that values of nuclear volume in blastema cells as well as in stump tissue cells were higher in *Xenopus than in Bufo.*

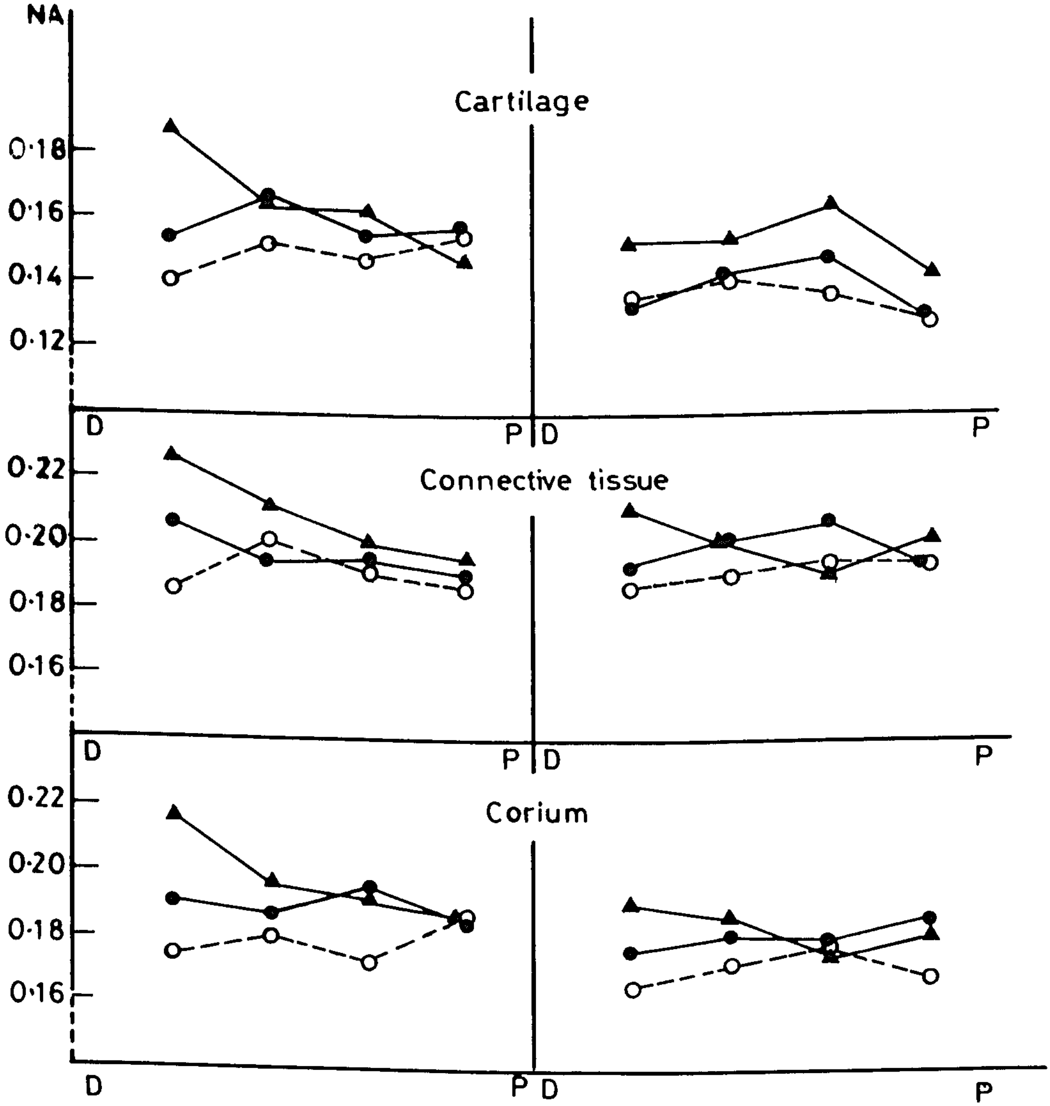

Fig. 4: Mean area of nuclear sections (NA) of cartilage, connective tissue and corium cells at stage 57 of *Xenopus*, along the proximodistal axis of experimental limbs (left), fixed 1,4 and 7 days after amputation, as well as of control limbs (right). Other abbreviations as in fig. 2.

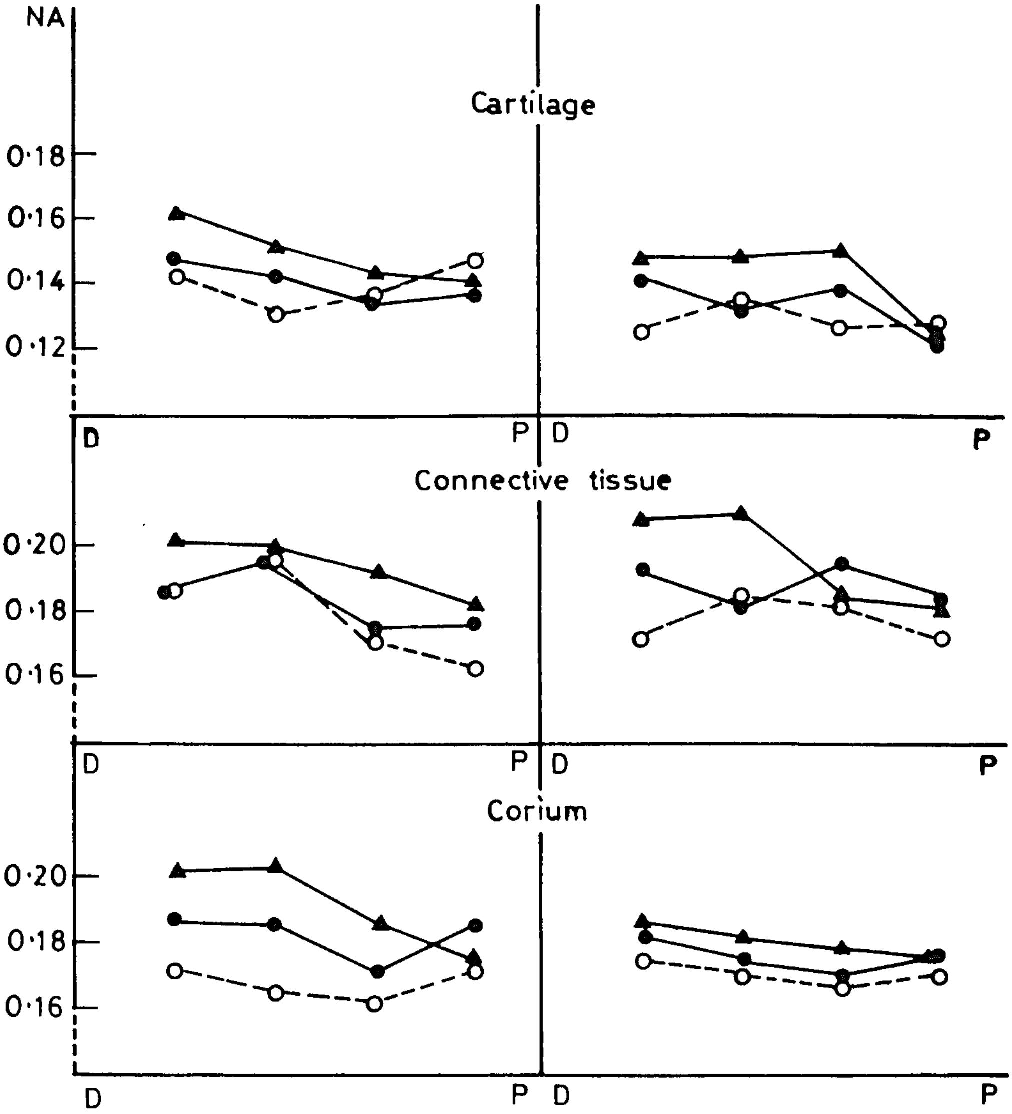

Fig. 5: Mean area of nuclear sections (NA) of cartilage, mesenchyme and corium cells at stage 53 of *Bufo,* along the proximodistal axis of experimental limbs (left), fixed 1,4 and 7 days after amputation, as well as of control limbs (right). Other abbreviations as in fig. 2.

Blastema and stump volumes

The regenerating limb increases in size with the progress of post-amputation days. This means that the volume of blastema and that of stump increased progressively after amputation. It was also observed in the present experiments that the regenerating hind limbs and the larvae themselves, were morphologically larger in *Xenopus* than in *Bufo* for the two concerned stages. Therefore, the value:

$$\frac{\text{Blastema volume}}{\text{Stump volume}} \quad \text{or Vb/Vs}$$

, was more acceptable to compare between volume values in *Xenopus* and *Bufo.*

From table 1, it was indicated that the volume of blastema and that of stump at stage 53 of *Xenopus* showed higher values than those of *Bufo.* The value Vb/Vs was also higher in *Xenopus.* This value decreased at stage 57 for both species, although at blastema and stump volumes increased. T-test analysis for probability revealed that there was a significant difference ($P<0.05$) in the value Vb/Vs between *Xenopus* and *Bufo* at atage 53, while no significant difference between them was obtained at stage 57. However, there was a significant difference ($P<0.05$) in that value for *Xenopus* between stage 53 and 57, but for *Bufo,* there was a little or insignificant difference ($P>0.05$) between the two stages.

Correlative relationship

The relatinship between the nuclear volume of blastema cells (mean value throughout the blastema proper) and the total volume of blastema was statistically anlysed and the correlation coefficients (C.C.) were calculated for both stages 53 and 57 of both *Xenopus* and *Bufo,* on the seventh post-amputation day. At stage 53, C.C. calculated for *Xenopus* was 0.8589 and for *Bufo* was 0.9949. These values are sufficiently large to indicate a linear relationship between the nuclear volume and blastema volume. However, at stage 57, the C.C. claculated for *Xenopus* was -0.2429 and for *Buf* was -0.0419. These values indicate no correlation between nuclear volume and blastema volume.

DISCUSSION

The nuclear volume in the stump tissues throughout the proximodistal axis of control limbs was nearly uniform. It increased by amputation during dedifferentiation and transformation of these tissues into blastema cells. This enlargement of the nuclei may indicate the increase in cell metabolic activity which may be considered as a necessity of tissue dedifferentiation. It was earlier demonstrated that the cells of early blastema appear to have nearly the same nuclear size as the older blastema cells (Hay, 1958). In contrast, our results revealed that, in the older blastema cells, formed by the seventh post-amputation day, at stage 53, the nuclear area (maximum, 25.37 μm^2, *Xenopus*) was much more higher than that in the early blastema established by the fourth day (maximum, 17.88 μm^2, *Xenopus*).

The proximodistal gradient in nuclear volume was clearly pronounced in the mesenchymal blastema formed at stage 53. The distal blastema region was occupied by cells having the largest nuclei among all limb tisue cells. This region may be, therefore, considered as the most active region of the regenerating limb. Such a gradient in nuclear size was also evident in the stump tissues and the highest values were observed in the distal regions, i.e. those close to the amputation plane. This result confirms the earlier observation of the enlargement of nuclei and nucleoli with the commencement of active nucleic acid synthesis during the transformation of formed cell types into "undifferentiated - appearing" blastema cells (Hay, 1958). It was also indicated that cells with large nuclei increase their metabolic activity (protein systhesis) relatively more than those with smaller nuclei (Anton and Darda, 1969; Anton and Elsen, 1979; Anton and Bourauel, 1982). An increase with proximosdistal gradient in nuclear volume in blastema and stump tissues during dedifferentiation and blastema formation, was recently revealed in regenerating hind limbs of larval *Xenopus* and *Bufo* after amputation at the thigh or shank level (Anton *et al.,* 1986).

From the previous morphological and histological studies on anuran regenerating limbs, it was clearly evident that *Xenopus* possesses higher regenerative capacity among the other anuran species (see Dent, 1962; Goode, 1967). The present observations provieded a statistical evidence confirming that conclusion.

Although the size of the regenerating limb increased by the time after amputation in both species, the volume of blastema relative to the stump volume at stage 53 was higher in *Xenopus* than in *Bufo.* The cells of this blastema showed also higher nuclear volume in *Xenopus.* The increase in blastema volume at stage 53 may depend on the increase in nuclear volume, since there is a positive correlation beween them. The increase in the number of blastema cells owing to the active cell divisions and the increase in the intercellular substance may also affect the total blastema size.However, at stage 57 in both species, there was no correlation between nuclear volume and blastema volume, indicating that the increase in the size of blastema might be due mostly to the increase of intercellular substance. The nuclear volume, and blastema volume relative to the stump volume were less than those at stage 53, indicating the decreasing of regenerative ability from stage 53 to stage 57. The proliferation of blastema cells at stage 57 might take its part in the increasing blastema volume. It was interesting to observe that the value

Vb/Vs at stage 57 of *Xenopus* was slightly higher than that of *Bufo,* although the blastema volume in *Xenopus* was much higher than in *Bufo.* It was earlier recorded (Singer and Craven, 1948) that the regenerate volume may increase, partly owing to cell division and partly to the transient edema. Chalkley (1954) concluded that the changes in cellular volume reflect an increase in cell number and variations in cell size in the connective tissues and the contiguous blastema. The level of amputation may also be of significance in governing the regenerate volume. From the second to the fourth day after amputation at shank or ankle levels of hind limbs of *Bufo regularis* (Abdel-Karim, In press), the regenarate volume was nearly doubled, while at thigh level, it increased by three times.

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حجم البلاستيما والحجم النووي في الاطراف الخلفية المتجددة في يرقات زينوبس ليفيز وبوفو رجيولارس بعد البتر في مستوى رسغ القدم

أحمد السيد عبد الكريم

في هذا البحث وجد أن بعد البتر في مستوى رسغ القدم للأطراف الخلفية في الضفدع ذو المخالب **زينوبس ليفيز** والضفدع **بوفو رجيولارس** أن هناك زيادة في الحجم النووي لخلايا البلاستيما وخلايا أنسجة الجدعة . وكانت الخلايا في المنطقة الطرفية (القمية) للبلاستيما لها أنوية ذات أحجام أكبر من أحجام أنوية خلايا جميع الأنسجة الأخرى على طول الطرف المتجدد . أما في الجدعة فكانت الزيادة في الحجم النووي موجهة إلى الجزء الطرفي أو البعيد قريباً من مستوى البتر ، وكان من الواضح وجود تدرج أو منحدر قريب ـ متباعد (أمامي ـ طرفي) في الحجم النووي . وتوضح هذه النتيجة أن البتر يعمل على زيادة النشاط الأيفي لخلايا أنسجة الجدعة ، وكانت هناك اشارة عامة على أن قيم الحجم النووي في حالة جنس **زينويس** أعلى منها في حالة جنس **بوفو** .

وقد أظهرت قياسات الحجم الكلي للبلاستيما وللجدعة على أنه بالرغم من أن حجم البلاستيما في الطور اليرقي المتأخر ٥٧ كان أكبر منه في حالة الطور اليرقي المبكر ٥٣ ، فقد كانت قيمة حجم البلاستيما بالنسبة لحجم الجدعة : ($\frac{\text{حجم البلاستيما}}{\text{حجم الجدعة}}$) أعلى بكثير في حالة الطور المبكر . وكانت هذه القيمة أعلى في جنس **زينوبس** عنها في جنس **بوفو** .

ووجد أن هناك علاقة ايجابية بين الحجم النووي والحجم الكلي للبلاستيما في الطور ٥٣ بينما كانت هذه العلاقة سالبة في حالة الطور ٥٧ . وقد دل هذا على أن حجم البلاستيما الميزنشيمية (التي تتكون في طور ٥٣) يبدو أنه انعكاس للحجم النووي أكثر منه للمادة البيخلوية .