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# Effect of Boric Acid Solution on Cartilage Metabolism

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**Pelvic cartilage of chick embryo was used to demonstrate that presence of boron in culture medium decreases synthesis of proteoglycans, collagen and total proteins but on the other hand increases the release of these macromolecules. However, when glucose concentration in culture medium is brought to 22mM, the synthesis decrease is no longer observed, whereas release increase persists. Proteins released into the culture medium included heat shock proteins (70 hsp) and tumor necrosis factor $\alpha$ (TNF$\alpha$). The amount of phosphorylated proteins was enhanced in presence of boron while endoprotease activity in cartilage and in culture medium was significantly augmented. The *in vitro* effects of boric acid may explain its *in vivo* effect on wound healing.** 

Blech et al. (1, 2) demonstrated that a 3 % boric acid solution improved dramatically the healing of deep wounds (with loss of substances). Patients who were treated with boric acid returned to a normal care unit about three times more rapidly (mean 20 days) than those having received conventional antiseptics, such as chlorhexidine or iodinated polyvinylpyrolidone (mean 55 days). The boric acid solution induced rapid wound granulation and angiogenesis, but this was not seen in wounds treated with dilute HCl solution having an identical pH. Although boric acid solution has many advantages, such as efficiency and low cost, its use is limited by the need to protect healthly skin, hence boric acid solution must only be used until granulation appears (1, 2, 3).

Boron is an essential trace element for vascular plants. It regulates hormones and controls second messagers such as calcium (4). It also influences membrane potential and proton movements (5). Hunt et al. (6) demonstrated that boron deficiency lead to poor growth and leg abnormalities in chicks. Changes in dietary boron, affect calcium and bone metabolism in animals and humans (7, 8). Boron also affects energy metabolism (9) and has been shown to increase the concentration of plasma steroid hormones in rats and humans (10, 11).

The present study was undertaken to investigate, *in vitro,* the effects of boric acid on proteoglycan, collagen and proteins synthesis. As boric acid affect angiogenesis *in vivo* in the same way as TNF$\alpha$, we have examined the release of TNF$\alpha$ in response to boric acid.

## MATERIALS AND METHODS

*Synthesis of proteoglycans (PG) and collagen in cartilage in the presence of boric acid.* All experiments were carried out on pelvic cartilages from chick embryo eleven-day-old (12). Cartilages were preincubated for 1 h at 37° C in minimum essential medium (MEM, Gibco BRL, UK) buffered to pH 7.2 with 20 mM Hepes and containing 1 mmol non essential aminoacids, 2 mmol L-glutamine, 200 IU/ml penicilline and 80 $\mu$g/ml streptomycine. Each test was made on eight cartilages incubated in 8 ml of the above medium with or without boric acid, pH being readjusted to 7.2 if necessary. Incubation was continued with appropriate tracers for 20 h at 37° C. PGs synthesis was measured by the incorporation of [$^{35}$S] $Na_2SO_4$, which evaluates sulfation, the last step of PGs synthesis, and incorporation of [$^{3}$H]-glucosamine, which estimates PG lenghthening. The final concentrations of the tracers were 0.37 MBq/ml [$^{35}$S] $Na_2SO_4$ (3.7 GBq/mole, Amersham) and 0.74 MBq/ml [$^{3}$H]-glucosamine (1.4 GBq/mole, Amersham). Collagen synthesis was determined by measuring [$^{14}$C]-proline uptake (1.85 MBq/ml,), (15,7 GBq/mole, Amersham). The incubated cartilages were heated for 15 min. at 100° C and rinsed twice (1 h and 15 h) with 0.15 M NaCl saturated with $Na_2 SO_4$, glucosamine or proline, depending on the tracer used. This was followed by seven rapid washes with distilled water. Each cartilage was dissolved in 0.4 ml of Soluene 350 (Packard) overnight and the radioactivity was counted. The cartilages were previously weighthed and the results were expressed in cpm/mg cartilage. The same experiments were performed with 22 mM glucose in culture medium.

*Synthesis of proteins.* The cartilages were cultured under the conditions described above but with 3.7 MBq/ml [$^{35}$S] cysteine (3.7 GBq/mole, Amersham).

*Measurements of macromolecules released into the culture medium.* The culture medium from each test incubation was centrifuged (800 g, 10 min.) and macromolecules were precipitated with 4 % (w/v) trichloracetic acid (TCA) at 4° C for 30 min. The precipitate was washed twice with 4 % TCA, dissolved in 200 $\mu$l 0.4 % desoxycholate/0.1 M NaOH and the radioactivity counted. The results were expressed as cpm/ml of culture medium.

Total protein concentration was determined in untreated cul-



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ture medium using the bicinchoninic acid protein assay kit (BCA, Sigma, USA).

*SDS-PAGE electrophoresis.* Culture medium from each test incubation was centrifuged (800 g, 10 min.) and dialysed against distilled water (Spectra/pur membrane MWCO : 1,000, Polylabo) for 48 h at 4° C with gently shaking, to eliminate boric acid and free radioactive tracers. The dialysed medium was then lyophilized and dissolved in 200 $\mu$l distilled water. Total proteins were separated by SDS-PAGE under reducing conditions. Samples were dissolved in 0.125 M Tris pH 6.95 containing 4 % SDS, 10 mM EDTA, O.1 % DTT and 0.01 % bromophenol blue. The stacking gel (5 % acrylamide, pH 6.95) and the resolving gel (13 % acrylamide, pH 8.9) contained 2mM EDTA. The running buffer being adjusted to pH 8.75, contained 2 mM EDTA, 25 mM Tris, 0.1 % SDS and 0.2 M glycine. Proteins were stained with 0.1 % Coomassie blue solution and autoradiographied (Kodak film) 15 days at −80° C.

*Immunoblotting for TNF$\alpha$ and hsp 70.* Electrophoresis gel and nitrocellulose film (0.45 mm, Amersham) were equilibrated with transfer solution (48 mM Tris, 0.13 mM SDS, 39 mM glycine and 20 % methanol). Proteins were transferred using semi-dry apparatus (Bio-Rad) and the nitrocellulose film was saturated with blocking solution (5 % w/v, Bio-Rad) and gelatine (2 % v/v). The nitrocellulose membrane was then incubated in rabbit-polyclonal anti-human TNF$\alpha$ antibody (diluted 1/250 in 0.5 ‰ PBS-Tween 20) (Polyclonal antibody, Genzyme IP 310) overnight at room temperature, or with anti-hsp 70 monoclonal antibody (Sigma, H5147) (diluted 1/5 000). The membrane was washed three times in PBS-Tween and incubated with second antibody diluted 1/16 000 in PBS-Tween 20 (Rabbit IgG conjugated to peroxidase, Sigma, A 0545) or with secondary antibody diluted 1/8000 in PBS-Tween 20 (rabbit monoclonal anti-mouse IgG conjugated to peroxidase, Sigma, A9044) for 1 h. The membrane was washed twice with PBS-Tween and once with PBS. Enzyme activity was revealed using 3 3′ diaminobenzidine "DAB" (Prolabo) and $H_2O_2$ as substrates.

*Collagenase treatment.* Lyophilized proteins from the media of cartilages cultured with [$^{14}$C]-proline were dissolved in 200 $\mu$l distilled water, and 20 $\mu$l were added to 20 $\mu$l of 0.1 M formic acid containing 0.2 mM acetate of sodium, pH 7.6. The protein solution was divided in two parts; one was treated with 100 $\mu$g collagenase (*Clostridium histolyticum*, Boehringer) overnight at 37° C. The proteins were precipitated by 4 % TCA and collected by centrifugation (2,500 g, 15 min.). The pellet was washed with 4 % TCA and dissolved in 0.4 % desoxycholate in 0.1 M NaOH. The incorporated radioactivity was counted.

*TNF radioimmunoassay (RIA).* Culture media were ultrafiltered through a 30,000 Da cut-off membrane (Amicon Diaflo YM 30) and the ultrafiltrates were dialysed against distilled water, lyophylized and dissolved in 200 $\mu$l of distilled water. The TNF$\alpha$ in these samples was assayed using a Biotrak kit (Tumor necrosis factor $^{125}$I assay system with amerlex IM magnetic separation, Amersham). The detection threshold was 0.76 fmol/ml and the intra-assay coefficient of variation was below 10 %.

*Measurement of protease activity.* Total protease activity was determined using a commercial kit (Boehringer) with fibrine used as substrate. Cartilages were grinded in 10 % TCA with a Potter, the mixture was centrifuged at 1,000 g for 15 min. and protease was evaluated on the supernatant and on redissolved culture media. 10 $\mu$l of each were loaded on a fibrine thin layer and incubated for 20 h at 37° C. The diameters of the lysis spots were measured and the amount of protease was determined by comparison to a standard curve prepared with trypsin.

*Phosphorylation of proteins.* This assay was effected into 24 well plate. The cartilages were first incubated (1/well) 24h at 37°C in 2 ml MEM supplemented as above without FCS. Eight cartilages were incubated in 2ml of medium with (0.25 % w/v) or without boric acid. Phosphorylation was initiated by addition of 0.37 MBq of [$\gamma$-$^{33}$P]ATP (111 TBq/mmol, Isotopchim) plus 100 $\mu$l unlabelled ATP (30 mg/ml) to each culture. The incubation continued for 6 h. The reaction was termined by heating and radiolabelled explants were washed with 0.15 M NaCl, incubated for 1 h with ATP (200 mg/ml) and washed several times with distilled water. The half cartilages were grinded in 10 % trichloroacetic acid and total proteins were precipated and collected after centrifugation. $^{33}$P-labeled proteins were counted and separeted by SDS-PAGE electrophoresis and autoradiographied.

*Electron microscopy of cartilages.* Samples (1-2 mm$^3$) were fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 2 hours, then rinsed with cacodylate buffer before being post-fixed in 1 % osmic acid for 2 h. Afterwards, samples were dehydrated in an alcohol series, ending with 3 × 20 min. in absolute alcohol, and then were treated by propylene oxide for 1 h, before finally being polymerised at 56° C for 2 days. Sections (600 −1 000 A°) were cut on a Ultracut (E Reichat-Yung) microtome, contrasted by uranyl acetate and examined in an electronic microscope (Elmiskop, Siemens 102).

## RESULTS

*Effect of boric acid on cartilage metabolism.* Boric acid decreased lenghtening of proteoglycan chains, as

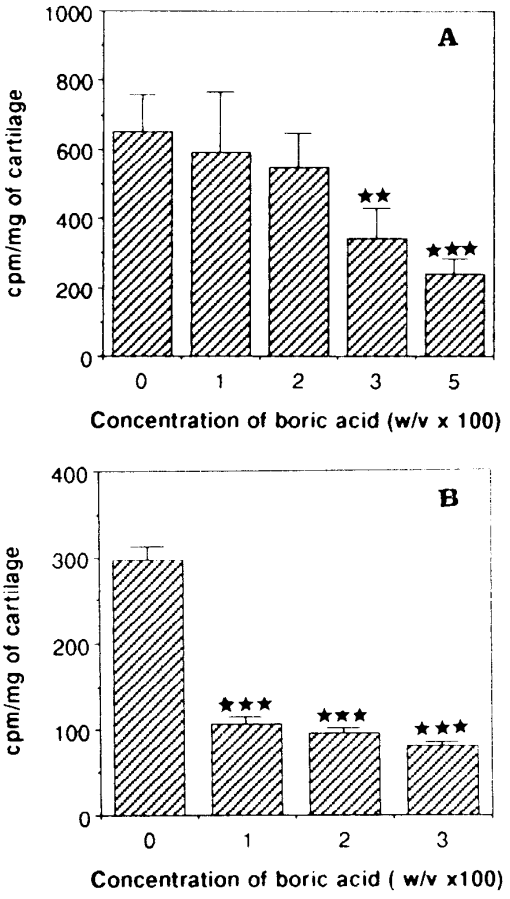


**FIG. 1.** Effect of boric acid solution on proteoglycan synthesis by pelvic cartilages of chick embryos. A: measurement of PG chain lengthening by [3H]-glucosamine incorporation, B: measurement of sulfation step by [$^{35}$S]-$Na_2SO_4$ incorporation. Each result is the mean ± SEM of eight replicates. ** $p < 0.01$; *** $p < 0.001$.

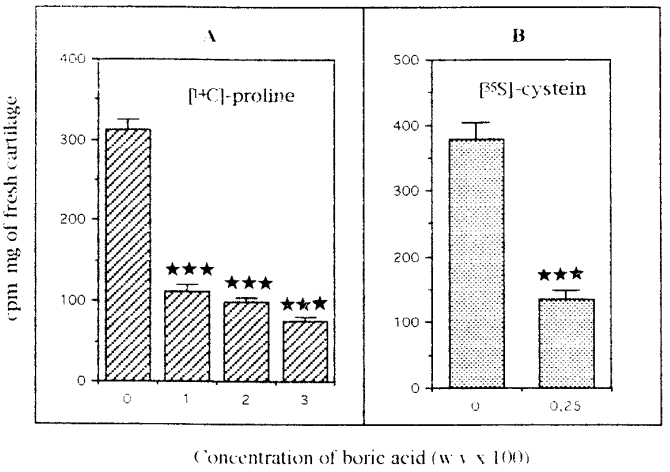


**FIG. 2.** Effect of boric acid solution on A: collagen, B: protein synthesis by pelvic cartilage of chick embryos. Each result is the mean ± SEM of eight replicates. *** $p < 0.001$.

judged by the incorporation of [$^3$H]-glucosamine, with a maximal effect (50 % of inhibition) at a 3 % solution (Fig. 1 A). The incorporation of [$^{35}$S] sulfate was equally dramatically inhibited by boric acid (Fig. 1B). Boric acid also decreased collagen synthesis-measured as [$^{14}$C]-proline incorporation (Fig. 2 A) as well as total proteins synthesis-measured as [$^{35}$S] cysteine, if it was present even at low concentrations (Fig. 2 B). However, the observed effects of boric acid might be resulted to its ability to complex glucose, so the same experiments were performed with 22 mM of glucose in culture medium instead of 5.5 mM. In thoses conditions, synthesis of PG and total proteins were not different from control (Fig. 3 A).

*Effect of boric acid on the release of macromolecules into the culture medium.* Boric acid stimulated release of proteoglycans into the culture medium (Fig. 4 A) as well as release of collagen. [$^{14}$C]-proline was well incorporated into collagen molecules, since the radioactivity disappeared after digestion with collagenase (Fig. 4 B). Furthemore boric acid solution increased total protein release from chick embryo cartilage over controls (Fig. 5). The amount of newly synthesized proteins released into the culture medium (maximum 172 %) was less than the total proteins (maximum 350 %).

The effect of glucose concentration in culture medium on release of macromolecules was also tested. Addition of boric acid at concentrations as low as 0.25 % increase release of macromolecules in supernatant with 22 mmol glucose in medium (Fig. 3 B).

As the increase in macromolecules released from cartilages in the culture medium could be explained by cell lysis due to boric acid toxicity, particularly in culture with 5 mM glucose, the cartilages were examined by electronic microscopy. The cartilage cells were undamaged, with intact membranes. The cells appeared also, to be secretory with well developed Golgi apparatus (Fig. 6).

Electrophoresis of total proteins shown very little protein in the control culture medium, whereas the medium from cartilages cultured in boric acid contained many bands. Autoradiography shown that the principal new synthetized proteins had apparent molecular mass of 37, 43 and 47 kDa. Proteins were also found with MM between 20 -14 kDa (Fig. 7 A).

Immunoblotting of medium from boric acid-treated cartilages with anti-hsp 70 monoclonal antibody, revealed a single band of molecular mass about 70 kDa (Fig. 7 B). Immunoblotting of medium from boric acid-treated cartilages with anti-human TNF$\alpha$ polyclonal antibody showed a single band of molecular mass about 17 kDa (Fig. 7 C). The TNF$\alpha$ measured by radioimmunoassay using a human TNF$\alpha$ polyclonal antibody was enhanced in culture medium from cartilage that had been cultured with boric acid [364 ± 4 fmol/ml (n = 3)] compared to 78 ± 3 fmol/ml (n = 3) in the controls.

As TNF$\alpha$ is known to stimulate protease activity, the total protease activity was measured in cartilages as well as in culture medium. Standard curves were traced out using trypsin, the regression equation of

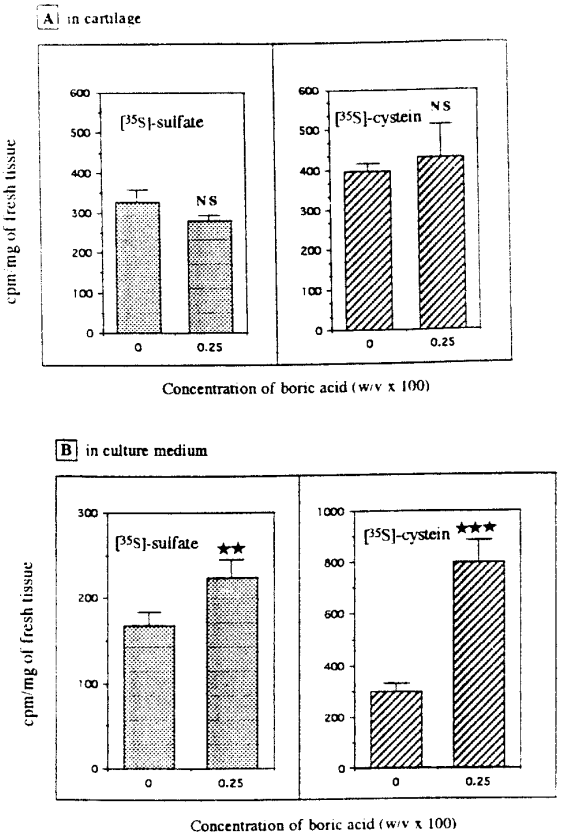


**FIG. 3.** Effect of boric acid solution on synthesis and release of proteoglycans and proteins in presence of 22 mM glucose in medium. A: in cartilage, B: in culture medium. ** $p < 0.01$; *** $p < 0.001$.

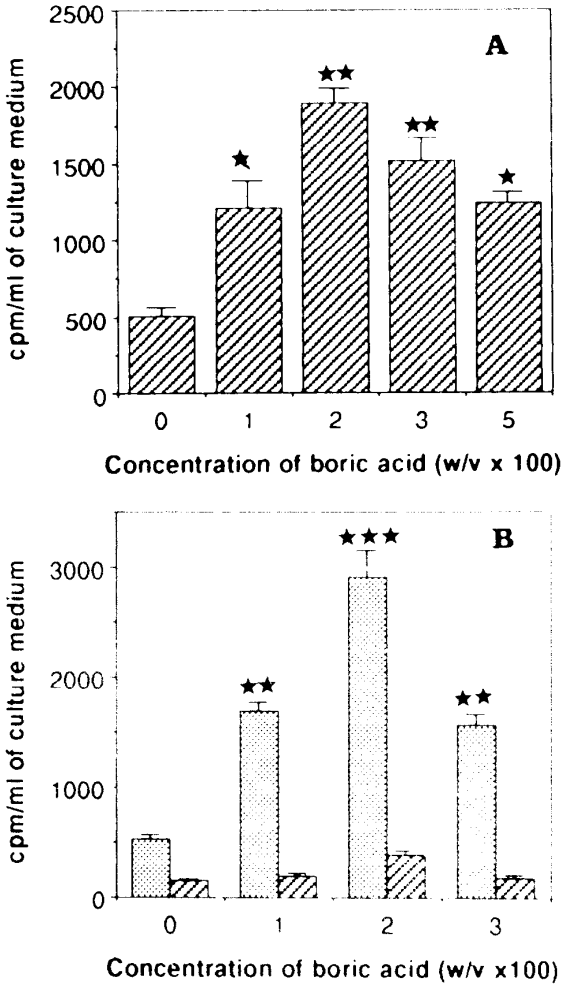


**FIG. 4.** Release of proteoglycans and collagen into the culture medium from pelvic cartilage of chick embryo. A: proteoglycans, measured as [$^3$H]-glucosamine, B: collagen, measured as [$^{14}$C]-proline, without ▨ and with ▨ collagenase. The results are the means of 5 assays. * $p < 0.05$; ** $p < 0.01$.

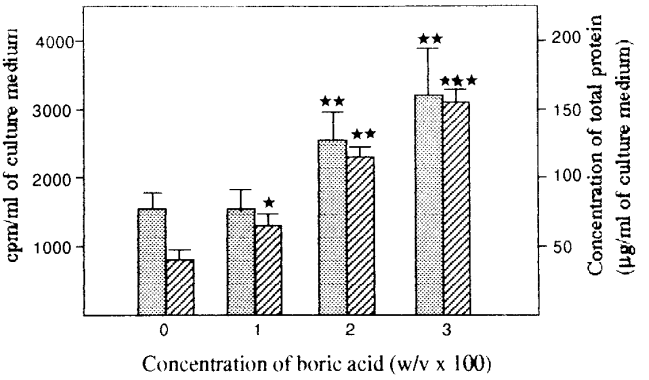


**FIG. 5.** Total proteins ▨ and newly synthesized proteins ▨ released by chick embryo pelvic cartilage, measured as [$^{35}$S]-Cystein incorporation. The results are the means ± SEM of three assays for total protein and five replicates for newly synthesized proteins. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

which was $Y = 2.44\ X - 0.34$ ($r = 0.997$). The protease activity was 3.6 ng and 8.1 ng/cartilage for control and assay (3 % boric acid); 1.27 and 2.19 ng/ml of culture medium.

Boric acid at low concentration enhanced significantly phosphorylated proteins (Fig. 8 A). The phosphorylated proteins had apparent molecular mass 7 kDa (Fig.8 B)

## DISCUSSION

The essentiality of boron for man was yet discussed, however there is a substantial evidence to establish it, although deficiency in human has not been yet demonstrated (13). Experiments of deficiency and supplementation in animals and man suggested that boron metabolism was closely linked to vitamin D metabolism. These two compounds play a role in calcium, phosphorus, magnesium metabolism (14, 15). Boric acid also accelerates wound repair *in vivo* (1). However, few is known concerning the role of boron on cellular level. As the deposition of connective tissue being a phase of wound healing process (16), we investigated *in vitro* the effect of boric acid on connective tissue metabolism using chick embryo pelvic cartilages in culture.

Boric acid solution decreased significantly the overall synthesis of proteoglycans and collagen, but it increased significantly the release of these compounds into the culture medium. These effects were not due to low pH, but to the boron itself, since all of the solutions were neutralized in culture medium and diluted HCl had no effect (data not shown). Boron being capable of forming complexes with polyhydroxy compounds (17), we examined whether observed effect was due only at the formation of glucose - boron complexes which would diminished energetic substrates for the cell. The synthesis of intracellular macromolecules was not modified when glucose was at 22 mM in culture medium. However, the increased release of these macromolecules in extracellular medium persisted. This was not related to boron membrane alteration since electronic

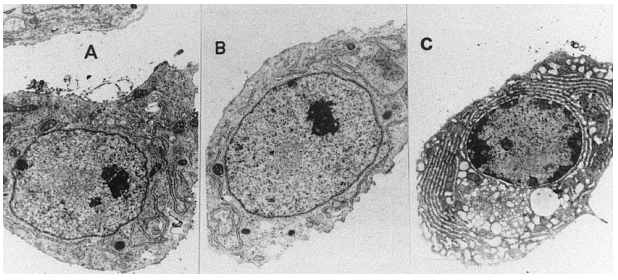


**FIG. 6.** Morphology of cartilage cells observed by transmission electronic microscopy. A: Day 0, B: After 20 h in MEM medium alone, C: After 20 h in MEM medium plus 3 % (w/v) boric acid.

microscopy shown that the cells had intact membranes and appeared to be secretory. But whatever we found about boron-glucose interactions *in vitro* with glucose decrease, we cannot related the *in vivo* action of boron on healing improvement since the tissus are always supplied with glucose by blood circulation. We, therefore, postulated that their secretion is an active process related to role of boron on calcium metabolism (18). These results are in contrast those with presented by Rajendran and al. (19) who observed that proline incorporation into collagen was accelereted and proteolytic enzymes were inhibited in the presence of amine carboxyborane. However, the corresponding amount of boron were lower than those we used.

Further an examination of the proteins synthetized and released was carried out. Boric acid also increased the amount of phosphorylated proteins, which were presented in a simple band of 7 kDa. The effect of boron on protein phosphorylation could be related to its influence on membrane characteristics or transmembrane signalling (20) but boron might merely inhibit the phosphatases. This effect jointed to the protease activity increase would explain why the found phosphorylated proteins had a low molecular mass.

The released proteins included hsp 70, which is induced by stress. Oberringer et al. (21) recently found a strong correlation between wound healing and production of hsp 70. The 14- 20 kDa bands may be of particular interest, because the effect of boron on angiogenesis *in vivo* mimics that of TNF$\alpha$ (22), and the molecular weight of TNF$\alpha$ is 17 kDa (23). TNF$\alpha$ is well present in the culture medium from cartilage cultured with boric acid. TNF$\alpha$ is known to act on cartilage metabolism. It stimulates resorption and inhibits synthesis of proteoglycans (24). Exogenous TNF$\alpha$ also inhibits collagen synthesis *in vitro* and *in vivo* (25), while endogenous TNF$\alpha$ is believed to down-regulate collagen synthesis during normal wound healing (26). TNF$\alpha$ induces fibroblast growth *in vitro* (27) and *in vivo* (28) and, lastly, fibroblasts respond to TNF$\alpha$ by producing several cytokines (29) which may also be involved in wound healing. However Bettinger et al. (30) and Kawaguchi et al. (31) found that TNF$\alpha$ impaired wound healing in vivo.

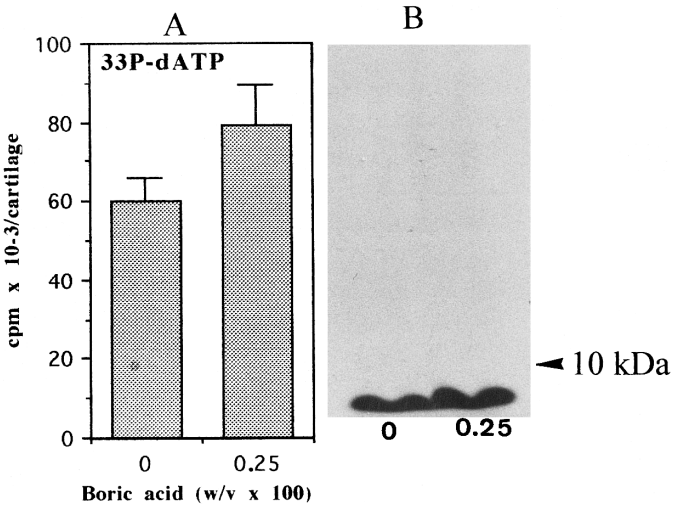


**FIG. 8.** Effect of 0.25 % boric acid on proteins phosphorylation: A; autoradiography of phosphorylated proteins: B.

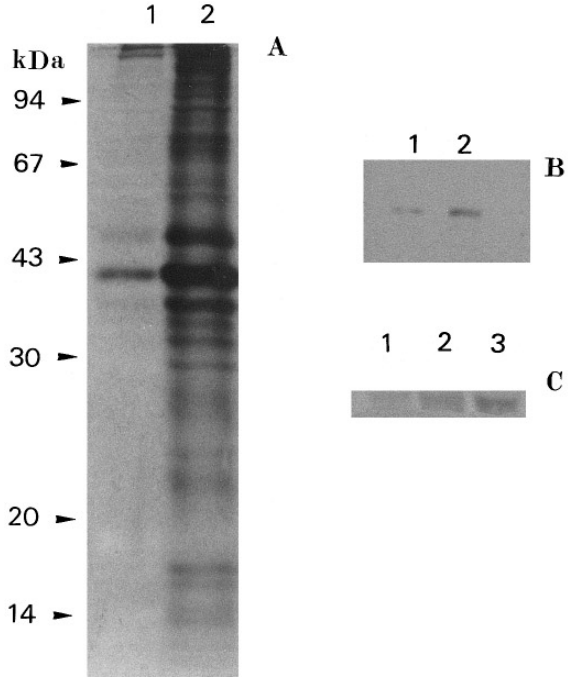


**FIG. 7.** A: SDS-Page electrophoresis and autoradiography, immunoblotting, B: hsp 70, C: TNF$\alpha$. A: 1 = without boric acid, 2 = with 3 % boric acid. B: 1 = without boric acid, 2 = with 3 % boric acid. C: 1 = without boric acid, 2 = with 2 % boric acid, 3 = with 3 % boric acid.

In this study it was demonstrated that cartilage cells release TNF$\alpha$ when treated with boric solution. Perhaps this phenomenon is the first of the healing process, and if it is, then it is necessary to know whether is only the release of preexisting (but complexed) TNF$\alpha$. The fact that endoprotease activity is increased would agreed with this hypothesis, or whether there is synthesis induction. The release of TNF$\alpha$ may, at least partly, explain the effect of boric acid on cartilage metabolism in vitro and on wound healing *in vivo.*

## ACKNOWLEDGMENTS

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