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Comparative calcification of native articular cartilage matrix vesicles and nitroprusside-generated vesicles

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Summary

Objective: Articular cartilage matrix vesicles generate calcium pyrophosphate- and basic calcium phosphate-like mineral *in vitro*. We sought to determine the morphologic features and calcifying capacity of sodium nitroprusside (SNP)-induced vesicles for comparison to those of controls.

Methods: Porcine articular cartilage was exposed to 1 mM SNP for 24 h and vesicles were isolated by enzymatic digestion and serial ultracentrifugation. Control vesicles were derived from an equal weight of untreated articular cartilage. Vesicles-containing fractions pelleted at 2×10^5 ***g*** · min (pellet I), 3×10^6 ***g*** · min (pellet II, the heavy vesicle fraction) and 8×10^6 ***g*** · min (pellet III, the light vesicle fraction) were analysed for Lowry protein content, nucleoside triphosphate pyrophosphohydrolase specific activity (NTPPPH) and ATP-dependent calcifying capacity.

Results: Electron micrographs (EM) revealed two populations of vesicular structures in both SNP and control pellets. In most experiments, there were no significant differences between the protein contents or ATP-dependent calcium accumulation of SNP vesicles compared to control vesicles. SNP vesicles in pellets I and II had lower NTPPPH activities than their respective controls, $P \leq 0.01$.

Conclusions: Our data confirmed that 24-hour treatment with the apoptosis-inducing agent did not increase matrix vesicle protein or alter the calcifying activity of vesicles compared to those from control cartilage. SNP did generate vesicles with lower NTPPPH specific activity in most experiments. SNP vesicles, although morphologically similar to controls, are not biochemically identical to them. © 2002 Published by Elsevier Science Ltd on behalf of OsteoArthritis Research Society International

Key words: Apoptosis, Matrix vesicle, Calcium pyrophosphate, Calcification.

Introduction

Apoptosis is a physiologic process of programmed cell death characterized by cell shrinkage, nuclear chromatin condensation, blebbing of plasma membranes and ultimately phagocytosis of the affected cells[1]. Recent studies examining degenerative cartilage for morphologic evidence of apoptotic cells suggest that apoptosis contributes to chondrocyte death and matrix degradation in osteoarthritis (OA). Human OA cartilage contains a significantly higher proportion of apoptotic chondrocytes than normal articular cartilage[2,3]. The number of apoptotic cells has been shown to correlate with the histopathologic grade of OA[4]. During experimental lapine OA, high levels of the chondrocyte apoptosis inducer, nitric oxide (NO), were elaborated from medial femoral and tibial plateau cartilage after anterior cruciate ligament transection. Apoptotic cells were noted in the superficial and mid zones of cartilage from ligament-transected rabbits and the prevalence of such cells correlated with nitrite production and the degree of gross morphologic cartilage fibrillation[5].

Recent studies also suggest that apoptosis is involved in pathologic calcium crystal formation in articular cartilage. Matrix vesicles (MV) are 50–150 nm membrane-bound subcellular components of cartilage digest isolated by high-speed ultracentrifugation. MV are an established nidus for calcification of epiphyseal cartilage[6]. MV isolated from articular cartilage generate either calcium pyrophosphate dihydrate- or apatite-like mineral *in vitro* in the presence or absence of ATP, respectively[7,8]. Membrane-bound structures have also been isolated from articular cartilage digest exposed to the apoptosis inducer, sodium nitroprusside (SNP)[9]. Other investigators[9] have termed vesicles derived from SNP-exposed cartilage apoptotic bodies (AB). AB derived from SNP-exposed human articular cartilage accumulate amounts of calcium similar to those precipitated by MV[9]. In addition, phosphocitrate inhibited SNP-induced porcine AB calcification to a degree comparable to that of native MV[10]. An ultrastructural comparison of isolated MV and AB has not been previously reported.

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We sought to determine whether SNP treatment of cartilage would increase the total amount of vesicles or change their enzymatic or calcifying capacity compared to vesicles from control cartilage by ultrastructural and biochemical analysis.

Materials and methods

All of the following reagents were obtained from Sigma Chemical (St Louis, MO) unless otherwise specified. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin-Fungizone (PSF), Hank's balanced salt solution (HBSS) and gentamicin were from Gibco, Grand Island, NY.

ORGAN CULTURE

Hyaline articular cartilage was shaved from the femoral condyles and patellae of freshly slaughtered mature pigs (Jacksonville Sausage Factory, Watertown, Wisconsin). Minced cartilage was washed once in DMEM with 10% fetal calf serum and 1% PSF (D-10-1). Equal wet weights of cartilage organ cultures were then incubated at 37°C for 24 h in D-10-1 with or without 1mM sodium nitroprusside. MV fractions derived from mature porcine, and normal and OA human cartilage all precipitate calcium in the presence of ATP, possess NTPPPH activity and show vesicle structures on electron micrographs[7–9,11,12]. The direct comparison of these characteristics between pigs and human has not been done. The large amount of cartilage required to perform the following experiments necessitated the use of porcine cartilage.

CARTILAGE DIGESTION AND VESICLE ISOLATION

Control and SNP-exposed organ culture underwent parallel digestion and isolation procedures to obtain control and SNP vesicle fractions, respectively. Control and SNP-exposed organ cultures were each sequentially treated with 0.1% (weight/volume) testicular hyaluronidase, type IV-S, then 0.5% (w/v) pancreatic trypsin, type II-S, then 0.2% (w/v) soybean trypsin inhibitor, type I-S, for 10 min each, at 37°C. Cartilage was then washed with HBSS without calcium and magnesium and digested with 0.2% (w/v) collagenase, type II, for 45 min and 0.05% (w/v) collagenase with 2% (w/v) lactalbumin hydrolysate for 12–16 h[13]. All reagent mixtures contained 1% PSF and the 0.05% collagenase mixture also contained 0.2% gentamicin.

Control and SNP-exposed cartilage digests were each centrifuged (International Equipment Co; Needham Heights, MA) at 10 000 ***g*** for 10 min to remove intact cells. Pelleted cells were filtered, resuspended in equal volumes of DMEM and counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). The 10 000 ***g*** supernatants were serially centrifuged in a Beckman Optima™ XL-100 K ultracentrifuge (Palo Alto, CA) at 20 000 ***g*** for 15 min to pellet large cell fragments (pellets I), at 50 000 ***g*** for 1 h to pellet heavy vesicle fractions (pellets II) and at 200 000 ***g*** for 45 min to pellet light vesicle fractions (pellets III)[11]. Each pellet was resuspended in 500 μl of DMEM and analysed for protein content by the method of Lowry[14]. Resultant vesicles are termed SNP and control vesicles.

ELECTRON MICROSCOPY

Glutaraldehyde, cacodylate buffer, osmium oxide, epoxy resin, urenyl acetate and lead citrate were from Electron Sciences, Fort Washington, PA.

Pellets I, II and III from SNP-exposed and control cartilage digests were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 and post-fixed with 1% osmium oxide. After dehydration, the pellets were embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi-600 transmission electron microscope (Hitachi Instruments Inc, San Jose, CA) at 100 kV. Vesicles were measured from electron micrographs (EM) using a Lupe magnifier. Vesicle sizes were grouped as follows: ≤75 nm, 76–200 nm and >200 nm.

NTPPPH ASSAY

Aliquots of each pellet were assayed in 100 μl of reaction mixture containing 50 mM HEPES buffered HBSS and 2 mM p-nitrophenyl thymidine monophosphate at pH 7.3. After incubation at 37°C for 2 h, the reaction was terminated by addition of 800 μl of 0.1 M NaOH. The p-nitrophenol product was estimated by measurement of the optical density at 410 nm. Quintuple determinations of NTPPPH activity were made on all samples and expressed as nanomoles of p-nitrophenol formed per hour per mg protein[15].

VESICLE CALCIFICATION

Calcification was assayed in a calcifying salt solution (CSS) containing 2.2 mM $CaCl_2$, 1.6 mM KH_2PO_4, 1 mM $MgCl_2$, 85 mM NaCl, 15 mM KCl, 10 mM $NaHCO_3$, 50 mM N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, and 1 mM ATP disodium salt, pH 7.6. Twenty-five-microliter aliquots of each pellet suspension was added to 500 μl of CSS, trace-labeled with 10^6 counts ^{45}Ca, vortexed, and incubated at 37°C[8]. Quintuple determinations were performed for each pellet. After 72 h, samples were centrifuged (Eppendorf; Brinkmann Instruments, Inc., Westbury, NY) at 10 000 ***g*** for 10 min. The supernatant was removed. The remaining pellet was twice washed with 500 μl of ice-cold CSS without ATP, and centrifuged after each wash at 10 000 ***g*** for 10 min. Accumulated ^{45}Ca was then solubilized in concentrated HCl and counted in a liquid scintillation analyser (Tricarb 2100TR, Packard; Meriden, CT). Amounts of accumulated calcium were expressed as a percentage of the total calcium in CSS and as μmol of Ca accumulated per mg of protein.

STATISTICAL ANALYSIS

The Wilcoxon signed rank and rank sum tests were used to compare paired and grouped data, respectively. All experiments were performed at least five times. Most experiments were performed 10 times. Results were expressed as mean± S.D.

Results

Exposure of cartilage organ culture to SNP resulted in a 15–80% reduction in the number of chondrocytes released

Table I
Electron microscopic analysis of vesicle sizes

Pellet	Vesicle type	Vesicle size±S.D. (no. of vesicles measured)	No. of micrographs
I	Control	120±26 (26)	6
		66±6 (10)	
	SNP	143±26 (18)	6
		53±14 (3)	
II	Control	113±23 (11)	15
		54±13 (19)	
	SNP	115±29 (11)	18
		56±15 (25)	
III	Control	49±6 (12)	12
	SNP	49±14 (10)	11

See Fig. 1 for methods.
Control=control vesicles.
SNP=SNP vesicles.

via enzymatic digestion compared to that of control cartilage thus confirming that SNP induces cell death.

ELECTRON MICROSCOPY

A total of 68 EM of control and SNP pellets I, II and III were reviewed. At least 6 EM were analysed per each vesicle type (Table I). EM were prepared from pellets obtained during one experiment. Two populations of membrane-bound vesicles (50–70 nm and 100–150 nm) consistent in size with murine epiphyseal vesicles[16] and articular cartilage vesicles[11] were identified (Table I). Measurements were taken only on those vesicles with discernable plasma membranes. Many more structures of similar size and density were noted within most EM (Fig. 1).

In addition to the two vesicle populations, pellet I, isolated at relatively low ***g*** forces, contained concentric membrane structures and larger (>200 nm) vesicle structures, the origins of which could not be determined. Pellet I from control organ culture digest also contained cells with pyknotic but intact nuclei. In contrast, no cells were seen in micrographs of pellet I from SNP organ culture digests. The SNP pellet I also contained fewer small vesicles (50–70 nm) than control pellets. This is probably due to random

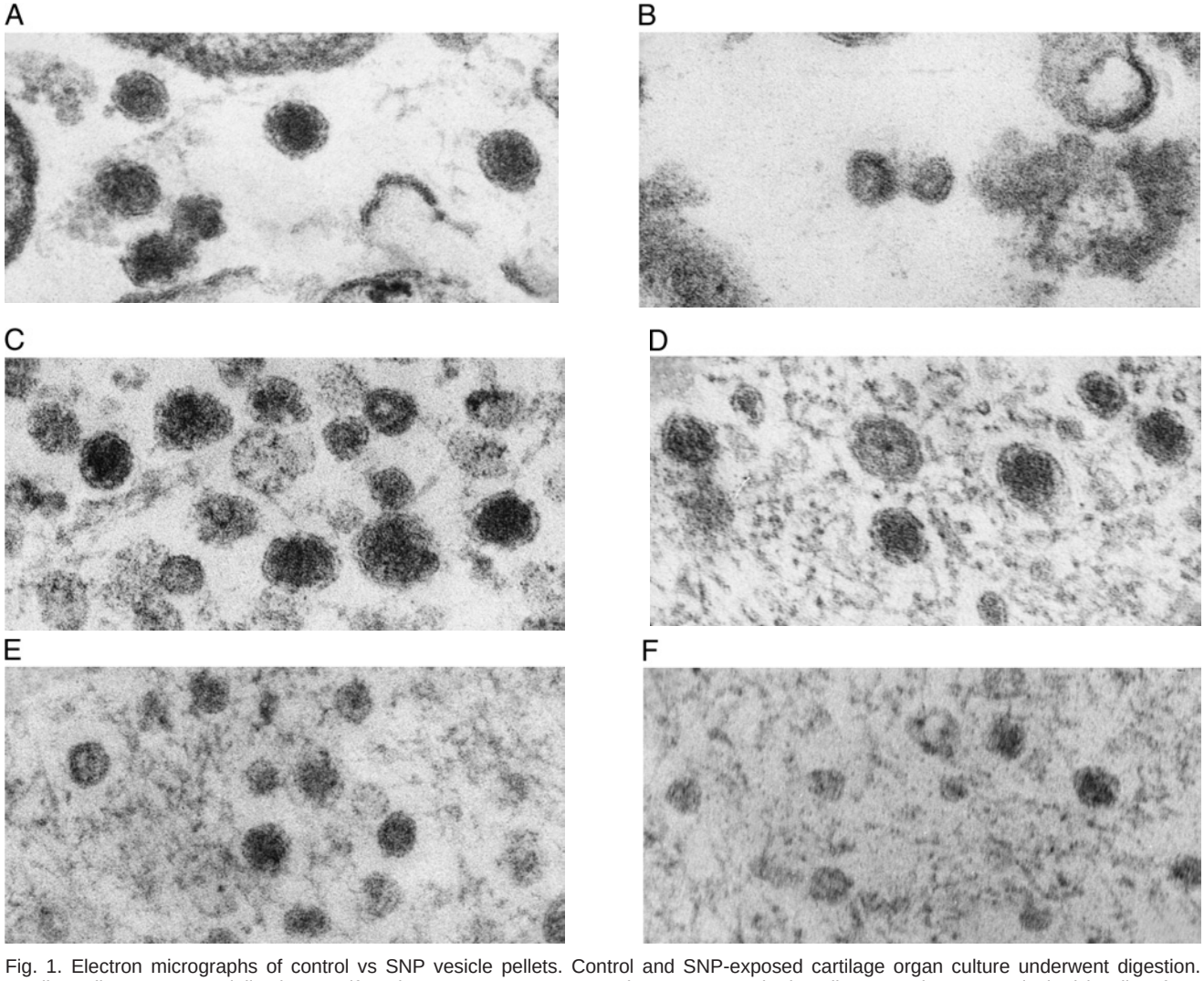

Fig. 1. Electron micrographs of control vs SNP vesicle pellets. Control and SNP-exposed cartilage organ culture underwent digestion. Cartilage digests were serially ultracentrifuged at 20 000 ***g***, 50 000 ***g*** and 200 000 ***g*** to obtain pellet I, II and III, respectively. (a) pellet I from control digest; (b) pellet I from SNP-exposed digest; (c) pellet II from control digest; (d) pellet II from SNP-exposed digest; (e) pellet III from control digest; (f) pellet III from SNP-exposed digest. Magnification 137 500×.

Table II
Statistical analyses comparing the Lowry protein contents of control vesicles vs SNP vesicles for each of pellets I, II and III

Pellet	Rank sum analysis of individual experiments No. of experiments			Signed rank analysis of pooled data
	NS	Control>SNP*	SNP>Control*	
I	6	2	2	NS
II	12	1	1	NS
III	10	1	3	SNP>Control*

Control and SNP-exposed cartilage organ culture underwent digestion. Cartilage digests were serially ultracentrifuged at 20 000 ***g***, 50 000 ***g*** and 200 000 ***g*** to obtain pellet I, II and III, respectively. Rank sum analyses of the Lowry protein contents of control pellets compared to those of SNP pellets revealed no significant differences in a majority of experiments.
NS=no significant difference between control and SNP vesicles.
Control=control vesicles.
SNP=SNP vesicles.
*$P \leq 0.05$.

Table III
Statistical analyses comparing the NTPPPH specific activities of control vesicles vs SNP vesicles for each of pellets I, II and III

Pellet	Rank sum analysis of individual experiments No. of experiments			Signed rank analysis of pooled data
	NS	Control>SNP*	SNP>Control*	
I	1	8	1	Control>SNP**
II	5	6	0	Control>SNP**
III	3	6	2	NS

Statistical analyses of the NTPPPH specific activities of control vesicles compared to those of SNP vesicles revealed that control vesicles possess higher NTPPPH specific activities in a majority of experiments. See Fig. 2.
NS=no significant difference between control and SNP vesicles.
Control=control vesicles.
SNP=SNP vesicles.
*$P<0.05$, **$P \leq 0.01$.

variations in the section of the pellet used for processing for electron microscope.

EM of pellet II from control and SNP organ culture digests each showed two vesicle populations. SNP pellet II EM also disclosed large (500–1000 nm) vacuoles while control pellet II EM demonstrated collagen fragments.

EM of pellet III from control and SNP digests showed only smaller sized vesicles. Control pellet III EM showed more uniform, electron dense vesicles than those of the SNP pellet III.

PROTEIN CONTENT

SNP exposure did not increase the protein contents of any of the three pellets compared to their respective controls in the majority of experiments (Table II). When differences between mean protein contents of SNP vs control pellets were analysed via the signed rank method, pellet III showed a higher protein content in only the SNP light vesicles (pellet III). The protein content from the final supernatants also did not show statistically significant difference.

Since it is impossible to quantitate the precise number of SNP and control vesicles, the Lowry protein content serves as a reproducible estimate of these quantities. The Lowry protein contents of SNP vesicles were not corrected for the number of remaining cells after SNP exposure because vesicles in those fractions may actually have come from damaged cells and should not be attributable to the remaining chondrocytes.

NTPPPH SPECIFIC ACTIVITY

In most experiments, exposure of chondrocytes to SNP decreased NTPPPH specific activity in all fractions (Table III). Comparison of mean specific activities from all experiments using the signed rank test confirmed a significant decrease in the SNP-treated group in pellet I ($P<0.01$) and II ($P=0.01$) but not pellet III.

Figure 2 shows the results of a representative experiment comparing NTPPPH specific activities of control and SNP vesicles.

CALCIFICATION ASSAY

SNP exposure did not increase the proportion of trace labeled calcium accumulated by pellets I, II or III in a majority of the experiments (Table IV). Furthermore, when calcium accumulation was expressed per mg protein, vesicles from all three SNP pellets showed similar calcium uptake to controls in most experiments (Table V).

Figure 3 shows the results of a representative experiment demonstrating the similarity of calcium accumulation per mg vesicle protein.

Discussion

Our study confirmed that treatment of cartilage with the apoptosis-inducing agent did not increase the total amount of vesicle protein available for calcification in most experiments nor did SNP treatment increase the calcifying activity of vesicles compared to those of control cartilage. SNP did generate vesicles with lower NTPPPH specific activity in most experiments. SNP vesicles did not calcify more than control vesicles but they were not biochemically identical to controls either.

In view of recent studies linking apoptosis with OA[2,3], it is tempting to ascribe the mineralizing properties of matrix vesicles (MV) to apoptotic bodies (AB). Were AB able to mineralize, they *might* serve as a nidus for formation of the pathologic minerals (calcium pyrophosphate and basic calcium phosphate) so commonly associated with OA[17]. Earlier experiments with vesicle fractions derived from SNP-exposed cartilage concluded that AB were biochemically and morphologically identical to MV[9]. Our study confirmed some of these similarities but did not explain the lack of an expected increase in vesicle protein or calcifying capacity under experimental conditions where apoptosis has previously been clearly demonstrated and where AB *should be* more plentiful. If AB are indeed the mineralizing nidus of OA cartilage, one might expect SNP pellets to have higher amounts of vesicle protein when compared to those of control cartilages. It is unlikely that the vesicle content of control cartilage was an artifact of the digestion procedure. Collagenase digestion of normal cartilage has previously been shown to induce only a 10% rate of apoptosis[2]. SNP organ culture in our experiments showed a 15–80% reduction in the number of chondrocytes compared to control.

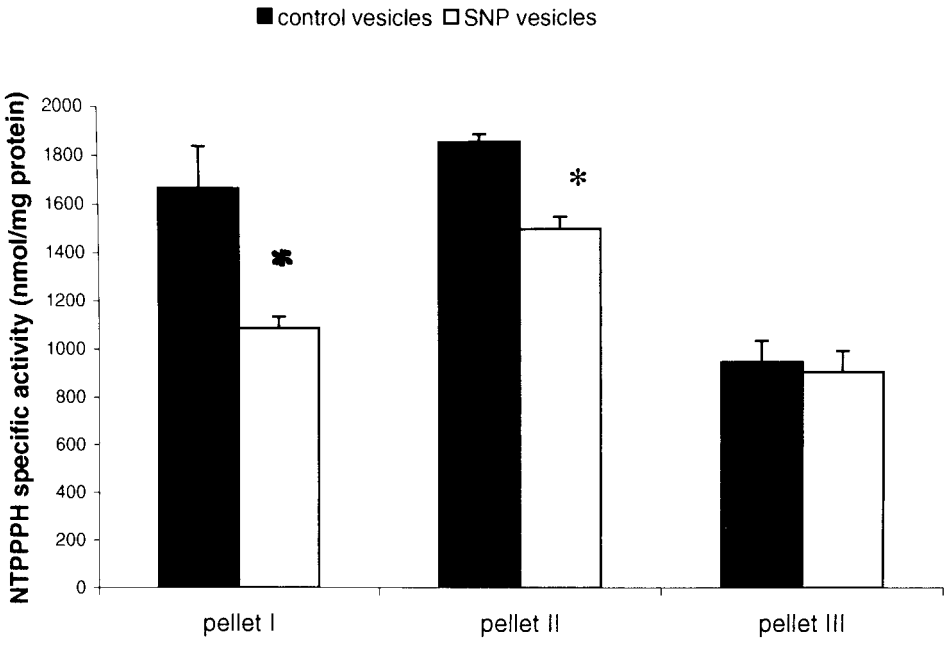

Fig. 2. Representative NTPPPH specific activities of control vs SNP vesicles. Control and SNP-exposed cartilage organ culture underwent digestion. Cartilage digests were serially ultracentrifuged at 20 000 ***g***, 50 000 ***g*** and 200 000 ***g*** to obtain pellet I, II and III, respectively. Aliquots of each pellet then were assayed in reaction mixture containing 2 mM p-nitrophenyl thymidine monophosphate at pH 7.3. The p-nitrophenol product was estimated by measurement of the optical density at 410 nm. Exposure of chondrocytes to SNP resulted in significantly decreased mean NTPPPH specific activities in pellets I and II. *=$P \leq 0.01$.

However, we did not find any more calcification in SNP pellets. AB do exist in normal cartilage. Lotz observed high number of apoptotic chondrocytes in the non-OA cartilages of some human donors aged 40–60[4] but this does not explain the striking lack of increases in SNP vesicle protein in the majority of our experiments.

It is possible that the procedures used in this and other previously published studies comparing isolated SNP vesicles to native MV have not succeeded in retrieving the majority of the SNP-induced vesicles. Pellet I, sedimented at the lowest ***g*** force in the serial ultracentrifugation protocol, had not been analysed in previous studies. Therefore, we examined *this* pellet in our studies expecting increased numbers of vesicles induced by SNP to be present in this fraction. There were, however, no significant increases in the vesicle protein or calcifying capacity of pellet I from SNP cartilage compared to that of control cartilage. It could be argued that SNP vesicles were mostly eliminated by the digestion procedure or were released into the initial organ culture media which was discarded at the beginning of the cartilage organ culture digestion procedure. Thus the similarity of SNP vesicles to constitutive isolated control vesicles noted here and previous reports may be due to the fact that the majority of vesicles induced by SNP exposure were not present in the isolated vesicle pellets studied which instead contained predominantly constitutive vesicles.

Future studies should examine the conditioned media from cartilage organ culture exposed to SNP in order to

Table IV

Statistical analyses comparing percentage of ^{45}Ca accumulation of control vesicles vs SNP vesicles for each of pellets I, II and III

Pellet	Rank sum analysis of individual experiments No. of experiments			Signed rank analysis of pooled data
	NS	Control>SNP*	SNP>Control*	
I	3	0	2	NS
II	6	1	1	NS
III	5	1	2	NS

Statistical analyses comparing the amount of calcium accumulated by control vesicles to that of SNP vesicles revealed no significant differences in a majority of experiments. See Fig. 3 for methods.

NS=no significant difference between control and SNP vesicles.
Control=control vesicles.
SNP=SNP vesicles.
*$P \leq 0.05$.

Table V

Statistical analyses comparing calcium accumulated per mg protein of control vesicles vs SNP vesicles for each of pellets I, II and III

Pellet	Rank sum analysis of individual experiments No. of experiments			Signed rank analysis of pooled data
	NS	Control>SNP*	SNP>Control*	
I	4	0	1	NS
II	5	1	2	NS
III	4	2	2	NS

Statistical analyses of the amount of calcium accumulated per mg protein of control vesicles compared to that of SNP vesicles revealed no significant differences in a majority of experiments. See Fig. 3 for method and graph.

NS=no significant difference between control and SNP vesicles.
Control=control vesicles.
SNP=SNP vesicles.
*$P \leq 0.05$.

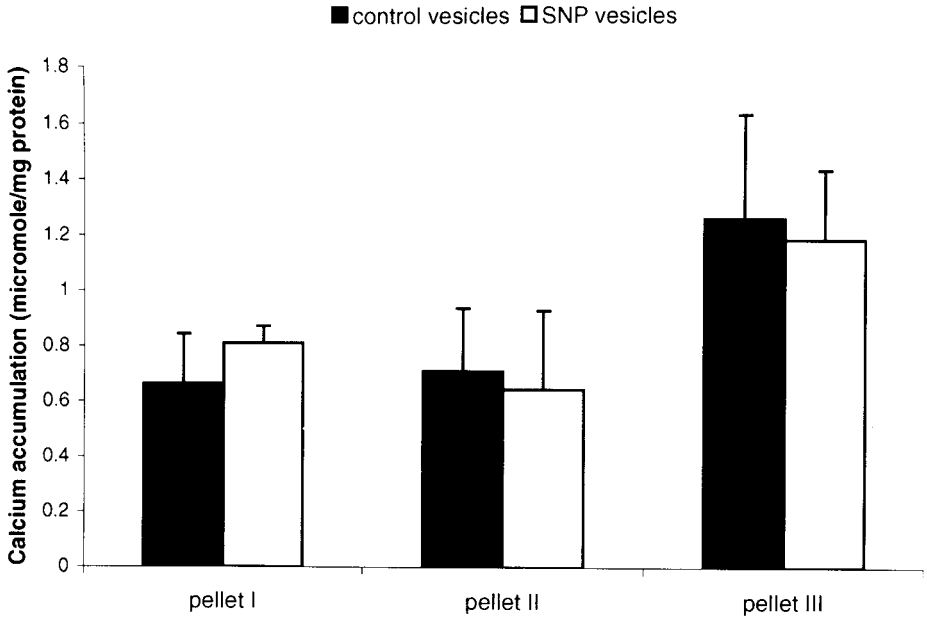

Fig. 3. Representative calcification of control and SNP vesicles. Control and SNP-exposed cartilage organ culture underwent digestion. Cartilage digests were serially ultracentrifuged at 20 000 ***g***, 50 000 ***g*** and 200 000 ***g*** to obtain pellet I, II and III, respectively. Calcification was assayed in a calcifying salt solution containing ^{45}Ca in the presence of ATP at pH 7.6. Accumulated ^{45}Ca was counted in a liquid scintillation analyser. There were no significant differences between calcification of control and SNP vesicles.

fully retrieve all vesicles induced experimentally and to analyse these for not only protein content and NTPPPH activity but also for their phospholipid profiles[18], associated macromolecules such as type X collagen[19] or annexin[20] and for other enzymes relevant to mineralization such as pyrophosphatase and alkaline phosphatase[21]. These studies should be compared to those performed on vesicles isolated from conditioned media of parallel control cartilages. We also recommend that these experiments should be repeated many times before conclusions are drawn, because batches of cartilage showed biologic variability in both enzymatic and calcifying assays.

In our studies, SNP vesicles accumulated calcium in the presence of ATP in amounts comparable to, but not exceeding, those of control vesicles. Once again, SNP vesicles did not show any more calcium uptake *in vitro* despite the marked reduction in the number of chondrocytes isolated from SNP-exposed cartilage. Qualitative analyses of SNP vesicle mineral are necessary to demonstrate whether similar mineral phases are formed by SNP vesicles compared to controls. Isolated MV derived from OA cartilage generate either calcium pyrophosphate dihydrate or basic calcium phosphate mineral under ATP-dependent and independent conditions, respectively[7]. Thus, in future studies of vesicles derived from conditioned media, calcium accumulation should be analysed in both the presence and absence of ATP and the resultant accumulated calcium should be subjected to further spectroscopic or diffraction analyses to determine whether SNP vesicles generate pathologically relevant mineral as do their MV counterparts. If so, further analyses of SNP vesicles may also reveal whether such crystals are more *or less* mature than those generated by MV. These studies would further distinguish the mineralizing role of SNP vesicles from that of constitutive MV.

In our study, the NTPPPH specific activity of pellet II from control cartilage exceeded that of SNP pellet II as analysed by the signed rank test but not the rank sum test. Since the signed rank test detected these differences by using data from all samples from all experiments, it is a more sensitive test. NO alters chondrocytes proteoglycan and glycoprotein synthesis[22] and theoretically may also decrease NTPPPH synthesis. We must, therefore, conclude that control and SNP vesicles isolated by our limited methods are not identical because of their differences in NTPPPH activity.

AB formed via differing mechanisms may demonstrate somewhat different enzymatic profiles or calcifying capacities. Hashimoto showed that a subpopulation of human OA and normal chondrocytes expressed the membrane protein Fas and are susceptible to Fas ligand-induced apoptosis. Although isolated AB from anti Fas-exposed chondrocytes generated similar amounts of pyrophosphate and accumulated similar amounts of calcium per mg protein compared to SNP-induced AB[9], direct comparisons of the Lowry protein content of these AB may demonstrate which method of apoptosis induction results in the greater total amount of calcification. Endogenous inducers of NO synthesis such as IL-1 may also result in AB with a unique enzymatic and calcifying profile provided that chondrocytes are simultaneously exposed to oxygen radical scavengers[23]. Because apoptosis is an ongoing process, the length of cartilage exposure to apoptosis inducers may also change the characteristics of retrievable AB and should be thoroughly studied.

Apoptosis is not the only form of cell death which chondrocytes may be subject to. Blanco[23] discovered that the chondrocyte's mode of death is likely to be determined by the balance between exposure to varying NO levels and levels of oxygen radicals. Increased NO level appears to be associated with chondrocyte apoptosis whereas increased levels of oxygen radicals are associated with chondrocyte necrosis. Further comparisons of the properties of vesicles derived from necrotic chondrocytes should be performed in order to discern which process, apoptosis or necrosis, results in greater calcification.

Finally, although it is possible that both AB and MV may coexist in differing proportions in control and SNP-exposed cartilage from mature pigs, the fact that isolated vesicles from both control and putative apoptotic cartilage calcify to a similar extent strongly suggests that *matrix integrity* may be the most important factor in MV and AB calcification. Proteoglycan (PG) depletion has been anatomically linked to chondrocytes apoptosis in human OA cartilage[4] and in lapine knee cartilage after anterior cruciate ligament transection[5]. Local matrix changes may trigger apoptosis[24] and/or result from apoptosis. In either case, extracellular matrix degradation is a key factor enabling cartilage mineralization[12,25]. MV have been isolated from normal articular cartilage of young persons but mineralized only in isolated form when released from the constraints of normal intact matrix[12]. Matrix degradation associated with apoptosis or necrosis may prove to be the most important determinant of *in situ* pathologic vesicle crystal formation.

In conclusion, treatment of porcine articular cartilage organ culture with SNP, an apoptosis-inducing agent, did not increase the amount of vesicle protein or alter the calcifying activity of isolated vesicles compared to that of isolated native control vesicles. SNP vesicles did possess lower NTPPPH specific activity in most experiments compared to that of controls. Further analyses of conditioned media may improve SNP vesicle retrieval. Local matrix degradation associated with apoptosis may constitute a more important link between apoptosis and calcification than changes in isolated vesicle numbers or activities.

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