Chondroprotective potential of root extracts of *Withania somnifera* in osteoarthritis

Venil N Sumantran[a,*], Asavari Kulkarni[a], Sanjay Boddul[a], Trushna Chinchwade[b], Soumya J Koppikar[a], Abhay Harsulkar[a], Bhushan Patwardhan[b], Arvind Chopra[c] and Ulhas V Wagh[a]

*[a]*Interactive Research School for Health Affairs (IRSHA), Bhartiya Vidyapeeth Deemed University Medical College Campus, Dhankawadi, Pune 411043, India*

[b]*Interdisciplinary School of Health Sciences, Pune University, Pune 411007, India*

[c]*Director, Center for Rheumatic Diseases, Camp, Pune 411001, India*

**Corresponding author (Fax, +20-24366929; venils@hotmail.com, vsumantran@gmail.com)*

This is the first report describing two novel chondroprotective activities of aqueous extracts of *Withania somnifera* root powder. First, these extracts had a statistically significant, short-term chondroprotective effect on damaged human osteoarthritic cartilage matrix in 50% of the patients tested. Second, these extracts caused a significant and reproducible inhibition of the gelatinase activity of collagenase type 2 enzyme *in vitro*.

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1. Introduction

Osteoarthritis (OA) is a serious degenerative disease that affects millions globally. Randomized, placebo-controlled, clinical trials performed between 1980 and 2002 confirmed the efficacy of oral glucosamine sulphate (GS) on arthritis (Richy *et al* 2003). However, controversies regarding the therapeutic efficacy of glucosamine in OA prevail (NCCAM 2006). Therefore, it is important to identify new chondroprotective drugs and nutraceuticals.

Roots of the plant *Withania somnifera* (*ashwagandha*) reportedly exhibit anti-inflammatory, antitumour, antistress, antioxidant, immunomodulatory, haematopoietic and rejuvenating properties (Mishra *et al* 2000). Although *ashwagandha* has been used for the treatment of OA (Kulkarni *et al* 1991), there are no reports demonstrating its chondroprotective activity *in vitro.*

We evaluated the chondroprotective potential of *W. somnifera* root powder in two assay systems. Studies on chondroprotective drugs report that proteoglycan (PG) release by cartilage explants is a proven marker of cartilage matrix damage *in vitro* (Nethery *et al* 1992). Therefore, we measured the effects of *W. somnifera* root powder on PG release from explant cultures of cartilage obtained from chronic OA patients at the time of knee replacement surgery. GS was used as a positive control, as it reproducibly reduced PG release from explant cultures of OA cartilage. Certain isoforms of matrix metalloproteinases express collagenase type 2 activity which degrades the cartilage matrix. Therefore, we also tested the effects of *W. somnifera* root on gelatinase activity of collagenase type 2.

This study provides the first *in vitro* confirmation of the chondroprotective efficacy of *W. somnifera* root powder in the treatment of OA.

Keywords. *Withania somnifera*; proteoglycan; collagenase; chondroprotection

Abbreviations used: OA, osteoarthritis; GS, glucosamine sulphate; PG, proteoglycan; HPLC, high performance liquid chromatography; CM, conditioned media; PG, proteoglycan

2. Materials and methods

Tissue culture plastic was obtained from Falcon Corporation, USA. Growth media, electrophoresis reageants, chondroitin sulphate (CS) and collagenase type 2 were from Life Technologies, Gibco, USA. GS capsules were from Nicholas Piramal Ltd, India. Reagents of analytical and cell culture grade were from Qualigens Corporation, India, and Sigma Chemicals, USA, respectively.

2.1 *W. somnifera root powder and preparation of extracts*

Dried roots of *W. somnifera* were collected and authenticated at the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (crude drug sample no. ERH/47). Powdered roots (powder A) were extracted with hot water and spray dried (powder B, extractive value 19.6%). Standardization was done by high performance liquid chromatography (HPLC) using withaferin-A and withanolide-A as reference standards. Powders A and B were solubilized in distilled water (10 mg/ml) by limited autoclaving (5 pounds pressure,7 min). These aqueous extracts were freshly prepared and filter sterilized (13 mm, 0.45 μm CN membrane) for each experiment.

2.2 *Profile of patients with osteoarthritis*

The selected patients (55–75 years of age) had suffered from chronic OA for 5–15 years prior to knee replacement surgery. Non-calcified, grade 1–2 articular cartilage from the lateral femoral condyles was used (Outerbridge 1961). The Outerbridge scale was used for classifying cartilage integrity. Definitions of cartilage integrity by this scale are given below:

Grade 1: mild softening or blistering of articular cartilage

Grade 2: Fragments/fissures in <1 cm^2 of the affected condylar cartilage
Both grades 1 and 2 are hyaline cartilage, with grade 2 being more brittle than grade 1.

Grade 3: Fragments or fissures in >1 cm^2 of the affected condylar cartilage

Grade 4: Cartilage erosion down to sub-chondral bone
Cartilage of grades 3 and 4 were not used in this study.

2.3 *Explant cultures of OA cartilage*

Explant cartilage cultures were set up within 1.5 hours of surgery, in 24 well tissue culture plates. The growth media was a 1:1 mixture of DMEM: Ham's F12 basal media with10% heat inactivated foetal bovine serum + gentamicin (8 μg/ml). After 1 day in culture, cartilage explants were treated for 24 h with/without sterile aqueous extracts of powder A or B (0.05 mg/ml). Explants were re-fed with growth media without herbal extract every 2 days for 8 days. Therefore, the drug was present only in the day 2 conditioned media (CM) samples. A CM sample from each explant was collected prior to each re-feeding, and stored at –20°C. All CM samples per OA patient were thawed and simultaneously assayed for PG levels.

2.4 *Chondroprotection assay*

2.4a Measurement of PG levels secreted by OA cartilage explants: Proteoglycan content was measured spectrophotometrically, using dimethylmethylene blue (DMMB) dye binding and CS as standard (Hoemann *et al* 2002). For each OA sample, triplicate wells (each with a cartilage piece/explant) were treated with *W. somnifera* extracts (powder A or B) for 24 h. For controls, triplicate wells of explants were treated with sterile distilled water for 24 h. For each patient, CM samples from control and drug-treated explants were assayed for PG content at each time point. Thus, CM from explants of each patient were assayed for total PGs at 4 time points (days 2, 4, 6, 8) post 24-h treatment with or without the drug.

2.5 *Analysis of data from chondroprotection assay*

Data for each CM sample are expressed as total PG content in microgram equivalents of CS/mg of explant/ml of CM at each time point for each patient; in the presence/absence of the drug. Raw data of PG levels in CM samples from explants at each time point per patient, treated with/without *W. somnifera* extracts, were tested for statistical significance using the Student 2-tailed *t*-test for paired samples.

We calculated the effect of the drug on PG levels in each CM sample, at each time point per patient sample using the ratio shown below.

$$\frac{\text{PG levels in CM from explants treated with drug}}{\text{PG levels in CM from control explants (treated with sterile water)}} \times 100$$

This ratio is expressed as per cent control PG release for each powder for each subset of patients. Values which showed a statistically significant difference from the corresponding controls (P<0.05) are shown in the responder subsets of patients. Data that lacked statistical significance are shown in both subsets of patients (responders and non- responders; figures 1 and 2).

2.6 *Collagenase type 2 assay*

An electrophoretic assay was used to measure the gelatinase activity of collagenase type 2 (Harsulkar *et al* 1998). A detailed protocol is given below.

2.6.1 *Resolving active enzyme protein by electrophoresis:* Pure collagenase type 2 (0.50% w/v or 5 mg/ml) was freshly prepared in enzyme buffer (50 mM Tris pH 6.8) and resolved on a non-denaturing 10% polyacrylamide gel without SDS (in stacking and resolving gels, running and sample preparation buffers). Unboiled gel samples were electrophoresed.

2.6.2 *Detection of enzyme activity:* Each lane was separately incubated with a different concentration of inhibitor solution (sterile aqueous extract of *W. somnifera* root powder A) or control solvent (distilled water) for 20 min on a shaker. Next, each lane was dipped into enzyme buffer (for 1–2 min) prior to being placed on an unused X-ray film (which contained a fixed concentration of gelatin). Exposure of each lane to the X-ray film for 30–60 min allowed visualization of clear bands of hydrolysed gelatin. The location of these "activity" bands coincided with the position of collagenase type 2 on native 10% gels (pure collagenase at 5 mg/ml reproducibly gave 2 bands.) Densitometry was done with a gel documentation system (Alpha Imager using Alpha Ease FC software, Alpha Innotech).

2.6.3 *Avoidance of artifacts in the collagenase assay:* The above protocol avoids artifact formation of an enzyme inhibitor complex for two reasons. First, there is no possibility of enzyme inhibitor complex formation at the origin of the gel, since the inhibitor is not present during electrophoresis. Second, an enzyme inhibitor complex cannot form during electrophoresis since the gelatin substrate is not embedded in the gel (as in zymography).

For these reasons, the observed inhibition of the gelatinase activity of collagenase by an extract of *W. somnifera* root powder A is genuine.

3. Results

3.1 *Validation of an explant model of cartilage damage using glucosamine sulphate*

In 5 of the 11 OA cases, the levels of PG release in response to GS decreased significantly with respect to the corresponding controls. Thus, the levels of PG present in GS-treated samples were 37.04 ± 25.75%, 53.67 ± 13.75%, 54.60 ± 11.29%, and 51.15 ± 13.93% of control values from CM samples collected at time points 1, 2, 3 and 4, respectively. In other words, treatment with GS of cartilage explants from these 5 cases caused a statistically significant 46%, 46% and 49% decrease in PG loss from these explants relative to corresponding controls at time points 2, 3 and 4, respectively. Thus, GS shows long-term chondroprotective activity in this cartilage damage model. (We define a long-term chondroprotective drug as one that causes a statistically significant decrease in cartilage damage [as measured by PG release] relative to the corresponding control in at least 3 of the 4 time points in this explant model.) These data validated our explant model and confirmed reports of GS's chondroprotective action *in vitro*.

Six of the 11 cartilage samples did not respond to GS. In these 6 cases, levels of PG release in response to GS averaged 120.91 ± 12.64%, 118.21 ± 29.81%, 121.67 ± 58.04% and 126.50 ± 34.85% of the corresponding controls at time points 1, 2, 3 and 4, respectively. These 4 values were not statistically significantly different from corresponding controls at these 4 time points.

These data are consistent with reports of a partial response to GS *in vitro* (Dodge *et al* 2003), and in clinical trials (NCCAM 2006) (*see* § 4.1.2).

3.2 *Chondroprotective effects of* W. somnifera *root in explants from OA patients*

Next, we studied the effects of *W. somnifera* root powder extracts on PG release from explants of the same set of 11 OA patients.

3.2.1 *Root powder A:* Figure 1A shows the PG release profile from explants of 3 of 7 patients who responded to *W. somnifera* root powder A (0.05 mg/ml). In these 3 cases, the level of PG release in response to powder A extract averaged 56.62 ± 20.12% of the control value at the first time point. These data were statistically significant, suggesting that powder A elicits a short-term chondroprotective response at the first time point. Figure 1A also shows that PG release in response to powder A was not statistically significantly different from corresponding controls at time points 2–4. Thus, levels of PG release in response to powder A extract averaged 81.02 ± 26.50%, 72.48 ± 49.55% and 111.24 ± 44.12% of the corresponding controls at time points 2, 3 and 4, respectively.

Figure 1B shows the PG release profiles of cartilage explants from the 4 cases that did not show any chondroprotective response to powder A (non-responders). In these 4 cases, levels of PG release in response to powder A extract averaged 121.13 ± 18.21%, 119.37 ± 34.88%, 114.23 ± 34.14% and 127.80 ± 50.07% of the corresponding controls at the time points 1, 2, 3 and 4, respectively. These 4 values were not statistically significantly different from corresponding controls at the 4 time points.

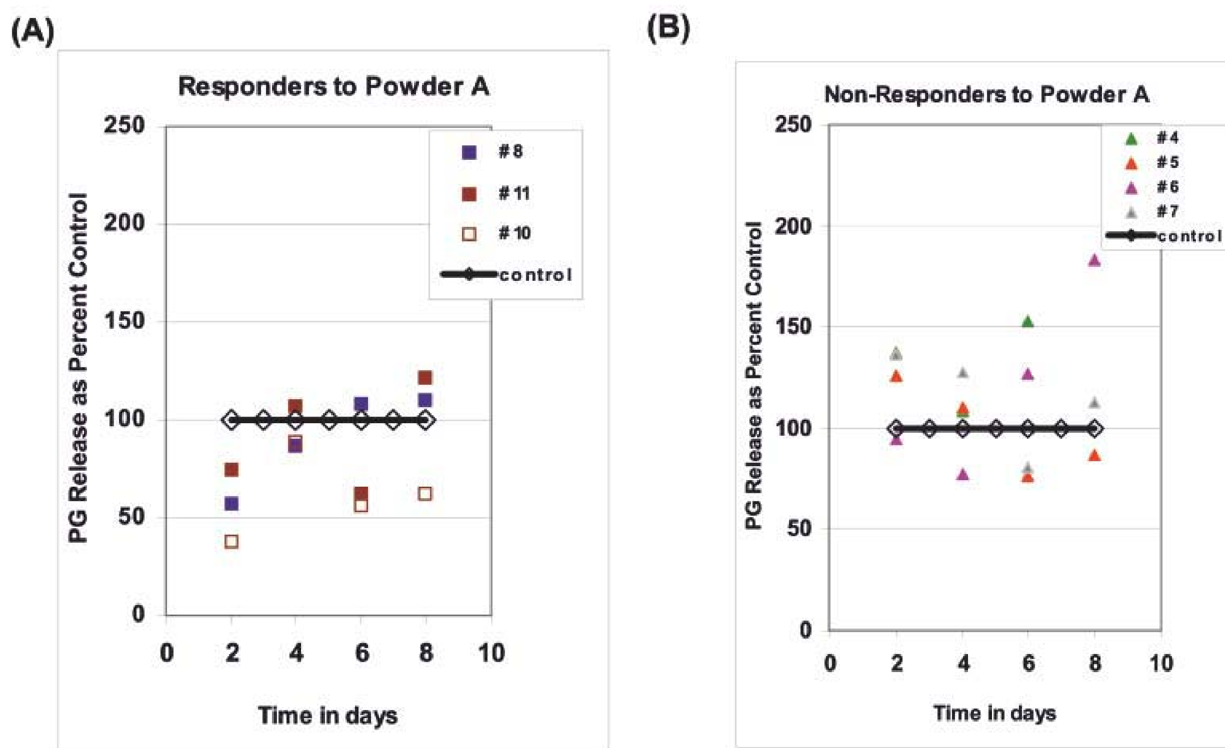

Figure 1. **(A)** Responders to *W. somnifera* root powder A. This graph shows the profile of proteoglycan release in 3 OA cases (cases 8, 10, 11) which gave a short term chondroprotective response to aqueous extract of *W. somnifera* root powder A. In these 3 cases, powder A caused a statistically significant decrease in the levels of PG release relative to corresponding controls. This was observed for the first time point only. The levels of PG release by controls for all samples is set at 100%. (**B**) Non-responders to *W. somnifera* root powder A. This graph shows the data for the 4 OA cases (cases 4, 5, 6, 7) which did not show a chondroprotective response to aqueous extract of *W. somnifera* root powder A.

3.2.2 *Root powder B: W. somnifera* root powder B (0.05 mg/ml) extract induced a statistically significant short-term chondroprotective effect in 6 of the 11 OA patients. In these 6 cases, the level of PG release in response to powder A extract averaged 65.31 ± 24.10% of the control at the first time point (figure 2A), suggesting that powder B (like powder A), also elicits a short-term chondroprotective response. Figure 2A also shows that the PG levels at time points 2–4 in response to powder B were not statistically significantly different from the corresponding controls at these time points. Levels of PG release in response to powder B extract averaged 100.96 ± 28.43%, 101.82 ± 22.59% and 115.55 ± 57.12% of the corresponding controls at time points 2, 3 and 4, respectively.

Figure 2B shows the PG release profiles of cartilage explants from the 5 cases that did not exhibit any chondroprotective response to powder B. In these 5 cases, the levels of PG release in response to powder A extract averaged 121.11 ± 28.47%, 108.17 ± 21.75%, 114.19 ± 27.04% and 101.95 ± 28.05% of the corresponding controls at the time points 1, 2, 3 and 4, respectively. These 4 values were not statistically significantly different from the corresponding controls at the 4 time points.

In summary, both *W. somnifera* root powders A and B exhibit short-term chondroprotective activity. (We define a short-term chondroprotective drug as one that causes a statistically significant decrease in cartilage damage relative to corresponding controls at the first time point in this explant model.)

3.2.3 *Testing for artifacts in the PG assay*

3.2.3.1 *Effects of* W. somnifera *root powders A and B on binding of CS to DMMB:* We did not find any precipitates in the test mixture CM samples from cartilage explants of any of the patients at any of the time points. This was true for CM samples from controls and those treated with drugs.

However, since we observed a short-term chondro-protective effect of both *W. somnifera* root powders in the day 2 CM samples of explants, it was possible that aqueous

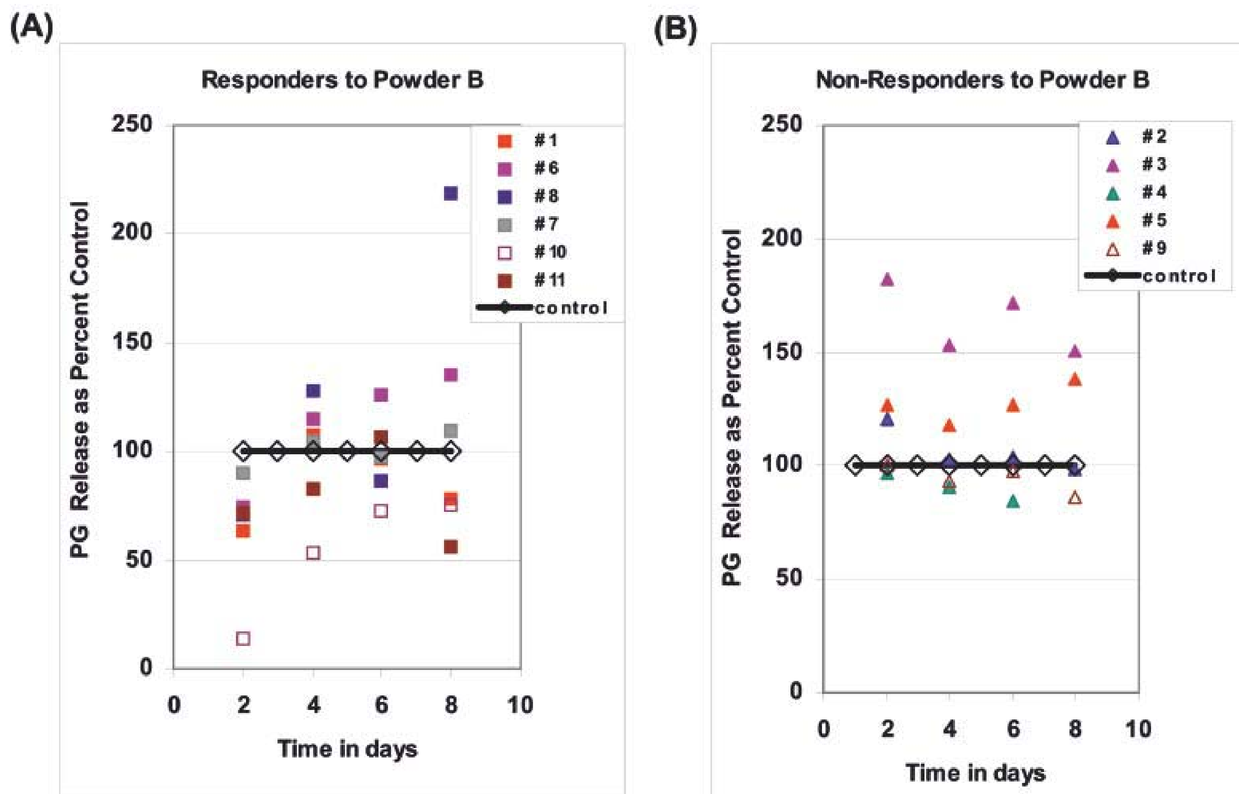

Figure 2. **(A)** Responders to *W. somnifera* root powder B. The graph shows the profile of proteoglycan release in 6 OA cases (cases1, 6, 7, 8, 10, 11) which gave a short term chondroprotective response to aqueous extract *W. somnifera* root powder B. In these 6 cases, powder A caused a statistically significantly decrease in the levels of PG release, relative to corresponding controls. This was observed for the first time point only. The levels of PG release by controls for all samples is set at 100%. **(B)** Non-responders to *W. somnifera* root powder B. This graph shows data for 5 of the 11 OA cases (cases 2, 3, 4, 5, 9) which did not show a chondroprotective response to aqueous extract of *W. somnifera* root powder B. The levels of PG release by controls for all samples is set at 100%.

extracts of *W. somnifera* root powders directly interacted with PGs and decreased their ability to bind DMMB. This would artifactually lower the PG signal to give a false-positive signal, i.e. it would appear as a chondroprotective effect. Therefore, the CS standard curve was done in growth media (DMEM Ham's F12 +10% FBS for explants) in the presence and absence of aqueous extracts of *W. somnifera* root powders A or B (0.05 mg/ml). Tables 1A and 1B show that these herbal extracts do not significantly alter the degree of binding between CS and DMMB, i.e. they do not artifactually interfere with binding of CS to the DMMB dye.

3.2.3.2 *Effects of* W. somnifera *root powders A and B on binding of complex PGs to DMMB:* Cartilage explants release simple and complex PGs of varying molecular weight. So, we also determined whether these herbal powders affected the binding of large PG molecules with the DMMB dye.

Cartilage explants from OA patient # 7 (control explants) were digested with papain to release PGs akin to those released in our experimental conditions (Tomasz and Jaworski 2000). Dilutions of 1:6- and 1: 24-fold of papain digests (in culture media) bound DMMB to give A540 nm values within the sensitive region of the CS curve. Briefly, each dilution of the papain digest was incubated in the presence or absence of aqueous extracts of both powders A and B (0.05 mg/ml) for 24 h in a CO_2 incubator at 37° C. The samples were then assayed for total PG levels.

Neither root powders A nor B significantly affected the affinity of papain digests for DMMB. Specifically, the A540 nm values of digests incubated with the powders were within 1–5% of A540 nm values obtained with these same papain digests lacking the herbal powders (data not shown).

3.2.3.3 *Chondroprotective effects of* W. somnifera *are not due to experimental artifacts of the PG assay:* In summary,

Table 1. Effects of aqueous extracts of *W. somnifera* root powder on binding of chondroitin sulphate to dimethylmethylene blue (DMMB)

A

Chondroitin sulphate concentration	Column 1 Chondroitin sulphate + powder A A540 nm	Column 2 Chondroitin sulphate alone A540 nm	Column 1 Column 2
1 mg/ml	0.150	0.143	1.049
0.5 mg/ml	0.128	0.128	1.00
0.1 mg/ml	0.027	0.027	1.00
0.05 mg/ml	0.018	0.021	0.857

B

Chondroitin sulphate concentration	Column 1 Chondroitin sulphate + powder B	Column 2 Chondroitin sulphate alone	Column 1 Column 2
1 mg/ml	0.133	0.143	0.930
0.5 mg/ml	0.114	0.128	0.886
0.1 mg/ml	0.021	0.027	0.778
0.05 mg/ml	0.019	0.021	0.904

Explant culture media were incubated with the indicated concentrations of chondroitin sulphate with or without 0.05 mg/ml *W. somnifera* root powder A (table 1A) or B (table 1B). The incubation conditions were identical to those used for explant cultures during the 24 h dosing period with drugs. Samples were then assayed for total proteoglycan by the DMMB assay. The table shows the raw spectrophotometric data (A540 nm values).

data in the previous section show that the chondroprotective effect of both the root powder extracts of *W. somnifera* reported in figures 1A and 2A are not due to direct binding or precipitation of root powders with simple CS or complex PGs. Therefore, the observed short-term chondroprotective effects are due to the ability of both the *W. somnifera* root powders to genuinely decrease the levels of PGs released by cartilage explants from 50% of the patients tested.

Furthermore, if the chondroprotective response of *W. somnifera* root extracts (powders A and B) were due to the artifacts discussed above, then one would expect this phenomenon to occur in the first CM sample (day 2) from all the 11 patients. However, only 6 of 11 patient samples exhibited a short-term chondroprotective response to powder B, and 3 of 7 patient samples gave a similar response to powder A. These data show that the short-term chondroprotective response of *W. somnifera* root extract is patient specific, and not due to an artifactual precipitate or complex between *W. somnifera* root extract and the PGs released from cartilage explants at the first time point.

Table 2. Summary of responders and non-responders to the drugs

Test drug	Total no. of cases	Responders	Non-responders
Glucosamine sulphate (GS)	11	5 (case# 1, 3, 8, 10, 11) showed LTCR	6 (case# 2, 4, 5, 6, 7, 9)
Powder B	11	6 (case# 1, 6, 7, 8, 10, 11) showed STCR	5 (case# 2, 3, 4, 5, 9)
Powder A	7 (of 11)	3 (case# 8, 10, 11) showed STCR	4 (case# 4, 5, 6, 7)

Cartilage explants from 11 OA cases were treated with/without glucosamine sulphate (GS) or the *W. somnifera* root powders (0.05 mg/ml) for 24 h. Proteoglycan levels were assayed in CM samples collected from explants at 4 time points post treatment with the drug (days 2, 4, 6, 8). Statistical analyses of these data revealed that explants from 5 OA cases showed a long-term chondroprotective response (LTCR) to GS, whereas explants from 6 cases showed no significant response to GS. Similarly, explants from six cases showed a short-term chondroprotective response (STCR) to *W. somnifera* powder B, whereas explants from the remaining 5 cases showed no significant response to it. Cartilage explants from 7 of the 11 OA cases were also tested with *W. somnifera* powder A (0.05 mg/ml). Explants from 3 of the 7 cases showed STCR to powder A. Explants from the other 4 cases showed no significant response to powder A.

3.2.3.4 *Summary of chondroprotection data:* The data on chondroprotective responses of cartilage explants from the 11 OA cases is summarized in table 2. *W. somnifera* root powders A and B (0.05 mg/ml) have similar chondroprotective potency in cartilage explants from 50% of the OA patients tested. Thus, 6/11 cases gave a short-term chondroprotective response to powder B, and 3/7 cases gave a similar response to powder A. Notably, explants from 3 of 6 OA patients showed a chondroprotective response to *W. somnifera* root powders A and B.

Interestingly, GS (0.05 mg/ml) and *W. somnifera* root powder B each induced a statistically significant chondroprotective response in 4 of the 11 cases (cases 1, 8, 10, 11). Table 2 also shows that explants from three patients (cases 8, 10 and 11) showed a chondroprotective response to all 3 test drugs (GS, and *W. somnifera* powders A and B).

The phenomenon of responders and non-responders to GS in table 2 is consistent with the largest clinical trial showing that GS alleviated pain in a small subset of OA cases (NCCAM 2006). In this context, our data showing that 6/11 of OA cases were "non-responders" to GS is not

surprising, since responsiveness of arthritic cartilage to GS (and drugs such as *W. somnifera*) may be absent in a high proportion of OA patients at the time of knee replacement surgery.

3.2.3.5 *Concentration of drugs and chondroprotective activity:* Interestingly, GS and *W. somnifera* root powder B lacked chondroprotective activity at 0.10 mg/ml (100 μg/ml). Thus, explants from 70–80% of the 11 patients did not respond to these drugs (data not shown). At this stage, cytotoxicity of this dose (0.10 mg/ml) of the 2 drugs in this explant model cannot be ruled out.

W. somnifera root powder A was an exception. This powder was tested in 7 cases at 0.05 mg/mg (table 2). In 5 of the 7 cases (cases 5, 6, 8, 10, 11), it was also tested at a higher concentration of 100 μg/ml (0.10 μg/ml). Powder A (0.10 mg/ml) induced a statistically significant short-term chondroprotective response in 3 cases (cases 5, 8 and 10). Levels of PG release in response to powder A (0.10 mg/ml) averaged 53.19 + 17.03% of the corresponding controls in these 3 cases at the first time point.

In summary, *W. somnifera* root powder A (not powder B or GS) gave a statistically significant chondroprotective activity in 2 of the 7 cases (cases 8 and 10) at both concentrations (*see* figure 1A, table 2). In spite of the small number of cases (n=3 for each concentration), the data on powder A (both concentrations) were statistically significant (P<0.05). This suggests that powder A has a biologically significant chondroprotective activity.

3.5 *Inhibition of collagenase type 2 by* W. somnifera *root powder A*

Based on visual observation, figure 3 shows that the minimum inhibitory concentration (MIC) of powder A for the enzyme (0.50% or 5 mg/ml) is 10 mg/ml (lane 2).

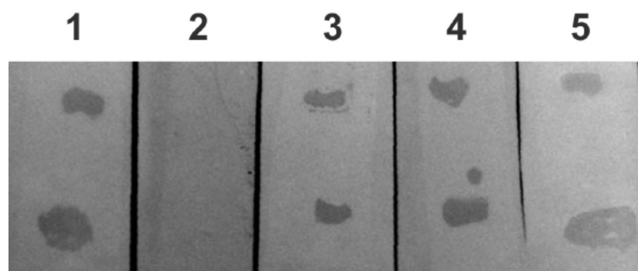

Figure 3. Gelatinase inhibition of type 2 collagenase by *W. somnifera* root powder A. Lane 1 shows activity of the enzyme (0.50% or 5 mg/ml) alone. Lanes 2–5 show activity of the enzyme after incubation with 10, 5, 2.5 and 1.25 mg/ml of aqueous extract of *W. somnifera* powder A, respectively. The minimum concentration of powder A (MIC value) required to inhibit the enzyme was 10 mg/ml (lane 2). Data are representative of triplicate experiments.

Densitometric analysis of the 2 bands (upper and lower) on the gel was done in order to detect possible dose-dependent inhibition of collagenase by *W. somnifera* extract.

With the density of the lower band of the enzyme control (lane 1) set at 100%, lanes 2, 3, 4, 5 gave values of 31.93%, 52.19%, 62.65% and 98.25%, respectively. With the density of the upper band of the enzyme control (lane 1) set at 100%, lanes 2, 3, 4, 5 gave values of 61.92%, 97.03%, 86.95% and 125.81%, respectively. Thus, figure 3 also shows the presence of dose-dependent inhibition of the gelatinase activity of collagenase type 2 by *W. somnifera* root powder A. As observed visually, the smaller isoform (lower band) of collagenase showed a clearer dose-dependent inhibition than the larger isoform (upper band).

Among the controls, collagenase type 2, a zinc metalloprotease, was specifically inhibited by the zinc chelator o-phenanthroline (MIC of 2 mM). Trypsin, a serine protease, was not inhibited by o-phenanthroline (data not shown).

4. Discussion

The key finding is that *W. somnifera* root powders (A and B at 0.05 mg/ml) showed reproducible, statistically significant, short-term chondroprotective activity in 50% of OA cases tested in an explant model of human OA cartilage damage (figures 1 and 2)**.** However, the remaining 50% of OA cases did not show a chondroprotective response to these powders (figures 1B and 2B)**.**

While it is true that a strict statistical approach demands averaging data on the chondroprotective effects of a given drug on cartilage explants from all 11 OA patients, we analysed the data of subsets of patients due to two important biological realities.

4.1 *Rationale behind statistical analyses of subsets of patient data*

4.1.1 *Unique nature of tissue sample:* Statistical analysis is done on random samples of data. Notably, the OA cartilage used in this study came from discarded joint cartilage of chronic OA patients undergoing knee replacement surgery. Such tissue cannot be considered a random sample, because it is very likely that such cartilage was chronically abnormal, and did not respond to drugs such as GS. In this context, it is significant that 50% of a small case number (11) showed a chondroprotective response to GS and *W. somnifera.* This may be because we used the least degraded cartilage (from the lateral femoral condyles). We were unable to discern any relationship between patient age, gender, state of cartilage and response to *W. somnifera* or GS. The drug history of each patient was unavailable due to patient confidentiality and ethical considerations.

4.1.2 *Heterogeneity of response to chondroprotective drugs*

(i) *Glucosamine:* As explained in section 3.1, clinical and *in vitro* studies point to the existence of responders and non-responders to GS. GAIT is the first, large-scale, multicentre clinical trial in the United States to test the effects of the dietary supplements glucosamine hydrochloride (glucosamine) and sodium chondroitin sulphate (CS) for the treatment of osteoarthritis of the knee (Clegg *et al* 2006). Of the 1,583 participants, 78% were in the mild pain subgroup and 22% in the moderate-to-severe pain subgroup. The primary outcome was defined as at least a 20% reduction in pain at 24 weeks. Data from the two subgroups were analysed. For the subset with moderate-to-severe pain, GS combined with CS provided statistically significant pain relief compared to placebo (about 79% had a 20% or greater reduction in pain versus about 54% for placebo). For participants in the mild pain subset, GS and CS, together or alone, did not provide statistically significant pain relief. The GAIT study is the best example of a partial response to GS. The researchers note that these findings are preliminary due to the small size of this subgroup.
(ii) *Ayurvedic drugs:* The phenomenon of responders and non-responders to drugs is even more true for ayurvedic medicine, wherein individual OA patients would be treated with different drugs according to body constitution, etc. Our data support this ayurvedic notion that a subset of OA cases respond to *W. somnifera,* because only a subset would have been given this herbal drug for OA. Thus, averaging the data from all 11 patients would have prevented detection of the existence of responders and non-responders to *W. somnifera and* GS. It would also have ignored new data of possible biological significance, i.e. the subset of OA cases that showed a short-term chondroprotective response to *W. somnifera* root powder. This information in itself is important and consistent with ayurvedic thought, other reports on GS, and pharmacogenomic studies identifying the genetic bases underlying differential drug response.

To summarize, analysis of subsets of data for statistical significance is unconventional. However, it is appropriate in this study due to the unique nature of the tissue sample and the known phenomenon of partial response to chondroprotective drugs.

These data on the chondroprotective effects of *W. somnifera* root powders on OA cartilage damage are novel and physiologically relevant for 3 reasons. First, we used only cartilage from chronic OA cases, since any chondroprotective drug must have a therapeutic benefit on degenerating human cartilage. Second, to mimic ayurvedic tradition, we only used aqueous extracts of crude *W. somnifera* root powders. Third, we used standard growth media (without special growth factors) for cartilage cultures. This approach allows examination of the effects of *W. somnifera* root powders complexed with natural serum factors. Thus, the observed bioactivities of these *W. somnifera* root extracts are likely to reflect their activity *in vivo*.

4.2 *Effective dose of* W. somnifera *root extracts in the two assays*

The chondroprotective activity of *W. somnifera* root powders in OA cartilage (figures 1 and 2) may in part be due to its inhibitory activity on collagenase (figure 3). However, the collagenase inhibitory dose of *W. somnifera* is 10 mg/ml, which is 20 times higher than the chondroprotective dose of *W. somnifera* root extract (0.05 mg/ml; figures 1 and 2). There are three probable reasons for this large difference in the effective dose of *W. somnifera* root extracts in the 2 assays which are discussed below.

First, it is entirely possible that very different molecule(s) within the aqueous extract of *W. somnifera* root exert the two observed effects, i.e. gelatinase inhibition and chondroprotection of OA cartilage. The second reason concerns the collagenase assay. Pure collagenase (0.50% or 5 mg/ml) is required for the detection of gelatinase inhibition by our method. Notably, the inhibitor has to diffuse through a 10% acrylamide gel in order to find the enzyme. Again, this enzyme-inhibitor complex must diffuse from the gel onto the X-ray film in order to block gelatin hydrolysis. It is therefore reasonable that the 10 mg/ml dose of *W. somnifera* root extract (MIC value) is required to inhibit 5 mg/ml enzyme. The third reason concerns the chondroprotection assay. Each cartilage explant (10–30 mg) is expected to express levels of collagenase protein in the microgram or nanogram range. Therefore, we expect a chondroprotective agent to be active in the μg/ml range. Notably, the chondroprotective dose of *W. somnifera* root extract and GS in this explant model of OA cartilage damage is 0.05 mg/ml or 50 μg/ml.

To summarize, there are scientific reasons which can account for the lack of relationship between the effective chondroprotective dose versus collagenase inhibitory dose of root extracts of *W. somnifera*. However, these two chondroprotective activities of *W. somnifera* are reproducible and physiologically relevant.

4.3 *Conclusions*

The chondroprotective drug doxycycline inhibited collagenase and gelatinase activities in cartilage from osteoarthritis patients *in vitro* (Smith *et al* 1998). Green tea polyphenols also inhibited pure gelatinase (Demeule *et al* 2000). In this context, the gelatinase inhibitory activity of collagenase by *W. somnifera* root powders is significant. This is particularly true since prior studies on *W. somnifera* root powders detected mainly its anti-inflammatory activity in animal models of joint disease (Mishra *et al* 2000).

Studies on the direct effects of this major ayurvedic *rasayana* on arthritic human cartilage and enzymes that degrade cartilage have not been reported before. The data in this pilot report are noteworthy, because they give the first preliminary evidence of the direct chondroprotective action of aqueous extracts *of W. somnifera* root powders on diseased osteoarthritic cartilage and direct inhibition of the gelatinase activity of collagenase type 2 by the same.

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