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Bart van Dijk, Esther Potier, Maarten van Dijk, Marloes Langelaan, Nicole Papen-Botterhuis, et al.. Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor.. Journal of Orthopaedic Research, 2015, 33 (11), pp.1724-1731. 10.1002/jor.22946 . hal-01758572

HAL Id: hal-01758572 https://hal.science/hal-01758572

Submitted on 4 Apr 2018

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Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor

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Key words: explant culture; disc herniation; inflammation; regenerative therapy; sustained release

To cite this article: van Dijk B, Potier E, van Dijk M, Langelaan M, Papen-Botterhuis N, Ito K (2015). Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor. J Orthop Res, 33(11): 1724-31. doi: 10.1002/jor.22946

Document Version: Accepted manuscript including changes made at the peer-review stage

Abstract: In intervertebral disc herniation with nucleus pulposus (NP) extrusion, the elicited inflammatory response is considered a key pain mechanism. However, inflammatory cytokines are reported in extruded herniated tissue, even before monocyte infiltration, suggesting that the tissue itself initiates the inflammation. Since herniated tissue swells, we investigated whether this simple mechanobiological stimulus alone could provoke an inflammatory response that could cause pain. Furthermore, we investigated whether sustained-release cyclooxygenase-2 (COX2) inhibitor would be beneficial in such conditions. Healthy bovine NP explants were allowed to swell freely or confined. The swelling explants were treated with Celecoxib, applied either as a bolus or in sustained-release. Swelling explants produced elevated levels of interleukin-6 (IL-6) and prostaglandin E2 (PGE2) for 28 days, while confined explants did not. Both a high concentration bolus and 10 times lower concentration in sustained release completely inhibited PGE2 production, but did not affect IL-6 production. Swelling of NP tissue, without the inflammatory system response, can trigger cytokine production and Celecoxib, even in bolus form, may be useful for pain control in extruded disc herniation.

Introduction

Low back-related leg pain, or radicular pain, is a common variation of low back pain. Its main cause is extruded lumbar disc herniation (1), during which the central core of the intervertebral disc, the nucleus pulposus (NP), pushes through the outer ring, the annulus fibrosus (AF). This extruded NP tissue can cause inflammation of nerve roots, which has been recognized as a key factor in painful extruded herniated discs (2).

Presence of inflammatory factors, interleukin-1 β (3,4) and 6 (5,6) (IL-1 β and IL-6), tumor necrosis factor alpha (3,4,7) (TNF α), and prostaglandin-E₂ (5,6) (PGE₂), is reported in herniated tissue. This is mainly attributed to the body's immune response: monocyte infiltration (8), macrophage maturation, and resorption of extruded tissue (9). But, production of several cytokines have been measured, albeit in lower quantities, in protruded NP tissue, which is not exposed to the body's immune response (6). Furthermore, Yoshida et al. (4) have observed IL-1 β and TNF α positive cells, before infiltration of monocytes. Thus, NP tissue itself may initiate

the inflammatory response through an unknown mechanism. We hypothesize that when NP tissue is extruded, it is exposed to a lower osmolarity and this osmotic shock in turn stimulates the native NP cells to produce inflammatory factors.

Radicular pain caused by herniation is generally treated conservatively, for example, oral analgesics, and 50% of patients recover spontaneously (10). However, in many patients, analgesia is insufficient (11), and they are treated with epidural steroid injections or surgery. Long-term results of surgery are not different from conservative treatment for radicular pain (12), and although epidural injections can be effective in 80% of cases, patients often need four to five injections within a year (13). Moreover, steroids might slow down the natural resorption of extruded NP tissue as shown in a rabbit tissue model (14). Reducing pain, while not inhibiting resorption, would be a promising approach to treat herniation.

One of the inflammatory factors in herniation is PGE_2 which can sensitize nerves and induce pain (15). Two enzymes are involved in PGE_2 production, cyclooxygenase 1 and 2 (COX1 and 2). Contrary to COX1, COX2 is inducible and, therefore, COX2 inhibitors are used in pain management, and have been shown to reduce pain in rat models of disc herniation (16). Celecoxib (Cxb) is a COX2-specific inhibitor and a candidate for treating herniated discs whose cells can produce PGE_2 , when stimulated by macrophages (2). However, because the half-life of Cxb is only 7.8 h (17), a biodegradable Cxb sustained release option is likely to be more successful than a single Cxb injection.

We have previously cultured bovine NP tissue explants in an artificial annulus, to prevent swelling and provide a near in vivo environment for the NP cells up to 6 weeks (18). In this study, we allow the tissue to swell, as in extruded herniation, and investigate if this simple mechanobiological stimulus can stimulate the tissue to produce cytokines, in the absence of the inflammatory system response. Two injectable sustained release biomaterials loaded with Cxb were then tested in this model and compared to a single Cxb injection.

Materials and Methods

Tissue

Tissue NP explants (150-350 mg) were isolated with an 8 mm biopsy punch (Kruuse, Sherburn, UK) from the center of fresh caudal discs of 24-month-old cows, obtained from a local abattoir in accordance with local regulations. After weighing, free swelling samples were immediately placed in 6 ml of culture medium (DMEM; Gibco Invitrogen, Carlsbad, CA), with 4 mM L-glutamine (Lonza, Basel, Switzerland), 1% penicillin/streptomycin (Lonza), 50 mg/l ascorbic acid (Sigma), and 5% fetal bovine serum (Gibco)). Control samples (non-swelling) were cultured in an artificial annulus system (18) in 6 ml of medium (Fig. 1). In this system, a jacket knitted from UHMWPE fibers (Dyneema, DSM, Heerlen, the Netherlands) lined with a 100 kDa molecular weight cut off (MWCO) semi-permeable membrane (Spectrum Laboratories, Breda, the Netherlands) prevents swelling.

Injectable gel

A hybrid thermo-reversible biodegradable hydrogel (Fig. 2) was used as one controlled release platform (TNO, Eindhoven, the Netherlands) (19). The hydrogel consists of a network of biodegradable nanoparticles linked to Lower Critical Solution Temperature (LCST) polymers. At room temperature, they are dispersed in water, injectable through a 32-gauge needle. However, at 37°C, they gel by crosslinks arising from hydrophobic interactions of the LCST polymers. The hydrogel was mixed with Cxb (LC Laboratories, Woburn, MA) in three different concentrations (12, 120, and 1200 mg/ ml gel). Previous *in vitro* experiments, showed that 0.5 ml gel, loaded with these concentrations, resulted in average releases of 0.1, 1, and 10 mM, respectively, when added to 6 ml of medium.

Microspheres

Biodegradable microspheres (DSM, Fig. 2) were prepared with an average particle diameter of 40 mm (20). During production, microspheres were loaded with 8.5% w/w Cxb. Previous *in vitro* experiments showed that 2.7mg of microspheres (loaded with 22.9 mg Cxb) in 6 ml of medium resulted in an average release of 10 mM. Therefore 2.7, 0.27, and 0.027 mg microspheres were added to a well to release 10, 1, and 0.1 mM Cxb.

Treatment conditions

Six NP tissue samples from independent donors were cultured in every experimental group (n = 6/group). Control artificial annulus samples remained untreated. Samples in free swelling condition were (1) not treated; (2) treated with a bolus (10 mM Cxb in medium for 3 days); (3) treated with a sustained release control (1 mM Cxb in medium for 28 days); and (4-11) treated with the microspheres or gel, loaded for an aimed release of: 0, 0.1, 1, or 10 mM Cxb (Table 1). Explants were cultured in 12-well deep-well insert plates (Greiner) with cell culture inserts (0.4mm pore size, Greiner) to hold the sustained release biomaterials. Samples were cultured for 28 days at 37°C, 5% O₂, and 5% CO₂ and medium was changed twice a week. During medium changes, temperature was kept at 37°C to prevent hydrogel liquefaction.

Biochemical content

Samples were weighed at the beginning and end of culture, and the percent increase in sample wet weight (ww) was calculated. Subsequently, they were stored frozen at -30°C, lyophilized overnight (Freezone 2.5; Labconco) and the dry weight (dw) was measured. The water content was calculated from ww-dw/ww. The samples were then processed as described earlier and used to determine their content of sulfated glycosaminoglycans (sGAG), hydroxyproline (HYP), and DNA, as well as the fixed charge density (FCD) (18). The amounts of sGAG, HYP, and DNA were expressed as percentage of the sample dw.

Cytokine release into the media

At every medium change, samples were collected and stored at -80°C. Release of cytokines into the medium was measured with ELISAs specific for bovine IL-1 β and IL-6 (both Thermo Fisher Scientific, Waltham, MA), specific for bovine TNF α (R&D Biosystems, Minneapolis, MN), and PGE₂ (Enzo Life Sciences, Farmingdale, NY) following manufacturer's instructions, and normalized to the original sample ww. All standard curves were dissolved in fresh culture medium, to account for any effects of the media on the analysis. Release was measured for all groups at days 3 and 28 as well as on days 14 and 21 for the best performing concentration of both loaded biomaterials and control groups.

Cxb concentration

At every medium change, samples were stored at -80°C, before Cxb concentration analysis by InGell Pharma (Groningen, the Netherlands). Samples were pre-treated as described before with slight modifications, that is concentration using liquid-liquid extraction with ethyl acetate, and mefenamic acid (Sigma) used as internal standard (21). The samples were analyzed with UPLC (1290 Infinity, Agilent, Santa Clara, CA) where the detection-limit was 10 ng/ml Cxb. The medium concentration of Cxb was measured for bolus treatment at days 14 and 28 and the best performing concentration of both loaded biomaterials at days 3, 14, 21, and 28.

Statistics

Matlab (Mathworks, Inc., Natick, MA) was used for statistical analysis. For all biochemical data, one-way analysis of variance (ANOVA) was performed, followed by Dunnett's test for differences compared to artificial annulus. For the cytokine release data of the selected groups, Kruskal-Wallis analysis was performed at each time point, followed by Bonferroni corrected Mann-Whitney post hoc tests for differences compared to artificial annulus. To investigate the difference in Cxb release between the two biomaterials, two-way ANOVA was performed, followed by Bonferroni corrected post hoc t-tests. Statistical significance in all cases was assumed for p<0.05.

Results

The ww of the artificial annulus group decreased 10% during culture, while free swelling groups increased between 100 and 150% ww (Fig. 3A). In all free swelling groups, the water content increased significantly (Fig. 3B), and the sGAG content (Fig. 3C) and FCD (Fig. 3D) decreased significantly compared to the artificial annulus group. In addition, the DNA content increased five-fold compared to the artificial annulus group (Fig. 3E). There were no differences in hydroxyproline content (Fig. 3F).

With both biomaterials, 1 mM aimed release was the lowest dosage that completely inhibited PGE_2 at day 28 (Fig. S1). These were analyzed at the intermediate time points (days 14 and 21), together with the artificial annulus, free swelling, sustained control, and bolus groups (Fig. 4). In artificial annulus samples, high levels of PGE₂ were produced during the first 3 days, but were ameliorated from day 14 onwards (Fig. 4A). Free swelling samples produced PGE₂ throughout culture, with maximal levels from days 14 to 28. Due to the large variance in response, these levels were only significantly different from artificial annulus at day 28. Bolus, sustained control and both biomaterial samples were significantly different from the artificial annulus at day 3, with a complete inhibition of PGE₂ production. From day 14 onwards, these groups were not different from the artificial annulus group, indicating continuous inhibition of PGE₂ production. In artificial annulus samples, very low or undetectable levels of IL-6 were produced throughout culture (Fig. 4B). In free swelling explants, significantly higher levels of IL-6 were produced, from day 14 onwards, which were maximal at days 14 and 21. In all treated groups, high levels of IL-6 were produced throughout the culture, except for gel 1 mM where IL-6 production was partially reduced at days 14 and 21 and not significantly different from artificial annulus. Very low or undetectable levels of IL-1ß and TNF α were produced in all groups and at all time points, and there were no differences between groups (Fig. S2).

Both biomaterials, loaded for an aimed release of 1 mM, released Cxb for 28 days (Fig. 5). The average release, determined from these four time points, was 0.84 and 0.68 mM for the microspheres and gels, respectively, that is, close to the aimed concentration of 1 mM Cxb release in the gel was stable around 0.7 mM for 28 days. A burst release was observed in microspheres during the first 3 days, which was significantly higher compared to the gel. Thereafter, the release decreased in the microspheres and was significantly lower at each subsequent time point. Cxb release at days 21 and 28 was significantly lower in the microspheres compared to the gel. Cxb was still measured in the culture medium of the bolus group at day 14 and even at day 28, and was significantly larger than 0.

Discussion

The artificial annulus prevented swelling, kept NP tissue stable (18, 22) and showed no long-term inflammatory response. Free swelling of NP tissue induced a sustained inflammatory response, of the inflammatory cytokine IL-6 and the nociceptive stimulus PGE₂. This supports our hypothesis that even without interaction with the immune system, the extruded NP may alone initiate and/or contribute to the inflammation seen in herniation. With its high tonicity, extruded NP tissue will absorb water and swell (22), causing a hypo-osmotic stress to the cells. Such a stimulus has been shown to also stimulate amnion derived cells in a COX2 dependent manner resulting in production of PGE₂ (23). NP cells

regulate daily changes in osmolarity through NFAT (5, 24) which is produced more in hypertonic conditions. Although NP cells in monolayer stimulated with hypo-osmotic stress demonstrated MEK/ERK and AKT involvement (25), further research is needed, to clearly understand the involved biological pathway(s), which may be exploited for alternative treatment strategies. Nevertheless, this simple mechanobiological stimulus, similar to the initial phase of extruded herniation, did consistently elicit an inflammatory response although the variance was large between samples. This could have been due to the differences in osmotic pressure between samples, but this will be even more variable in patients with their different states of disc degeneration.

PGE₂ is involved in painful herniated discs (15); thus, the observation that NP tissue itself is able to produce PGE₂ shows the promise of COX2-specific treatment for radicular pain. The role of IL-6 in extruded herniation is not clear. It may be beneficial to alleviate the negative effects of herniation by contributing to resorption of herniated tissue via upregulation of matrix metalloproteases (26), inhibiting proteoglycan production (26), and stimulating macrophage maturation (27). On the other hand, as it can induce hyperalgesia in rats (28), it may be detrimental as well. However, IL-6 can induce PGE₂ (29) so, it is possible that hyperalgesic effects of IL-6 were not direct but due to increased PGE₂ production. This ambiguous role of IL-6 in herniated disc disease should be further investigated, but if it is mostly beneficial, treating painful herniated discs with Cxb could be a promising alternative to currently used steroids that have been reported to slow down the natural resorption of extruded NP tissue in a rabbit tissue model (14).

Interestingly, swelling healthy bovine NP tissue did not directly produce effective doses of TNF α and IL-1 β , although both have been detected in herniated human tissue (3,7) and rabbits (4). However, similar to our results, culture of such specimens did not lead to presence of either cytokine in the medium, even after lipopolysaccharide stimulation (5). This could be because TNF α detected in homogenates of human herniated NP tissue was only membrane bound and no soluble TNF α was found (7). Furthermore, expression of these cytokines is associated with increasing degree of disc degeneration (31), but can also be produced by activated macrophages (32) neither of which were present in our system. Low levels of TNF α , though, can have already strong effects in NP-like tissues (33) and TNF α inhibitors have the potential to stop radicular pain in patients (34). Thus, the roles of TNF α and IL-1 β in painful herniation remain pertinent.

The DNA content in free swelling samples increased five-fold compared to artificial annulus. However, the PGE2 and IL-6 production at day 28 increased 30-90-fold, respectively, thus, cannot be explained by the increased cell number alone. In a previous study (22), increase in DNA was attributed to cell cloning and formation of a fibrous layer at the sample periphery. In the present study, we did not investigate the contribution of this fibrous layer to the production of IL-6 and PGE₂. As this fibrous layer most likely contains active dedifferentiated cells, its production of inflammatory factors might be considerable. However, the increased PGE₂ and IL-6 production in the first 3 days, during absence of the fibrous layer, indicate that NP cells within the tissue produce inflammatory cytokines as well. Furthermore, in vivo this layer will probably also be formed (35) and may initiate the formation of the granulation tissue seen in sequestered and extruded surgical samples (30). Any contribution of this layer to the production of cytokines in this study will occur *in vivo* as well.

Artificial annulus samples also increased PGE_2 production but only for the first 3 days. This is most likely a result of the shrinkage step needed before placing samples in the system. In this step, samples received a strong hyperosmotic stimulus (50% PEG in PBS (w/v) for 100 minutes), which has been shown to induce COX2 in renal cells (36). Besides this temporal production of PGE₂, no other cytokines were produced, showing that healthy bovine NP explants do not produce inflammatory cytokines, unless they are triggered.

Cxb was able to completely inhibit elevated PGE_2 levels for 28 days, when delivered continuously at a concentration of 1 mM in the sustained control group. This result was expected, as this is in the order of the therapeutic plasma concentration of Cxb (17). Both biomaterials were able to release Cxb continuously for 28 days and, thus, inhibit PGE₂. However, the release kinetics from the two biomaterials were significantly different, that is, microspheres showed a burst release, and a slowly declining release afterwards as observed earlier (20), while the gels showed constant release for 28 days.

To our surprise, the 10 mM bolus was also able to inhibit PGE₂ production of swelling NP explants for 28 days. This dosage is 10 times higher than the biomaterials and 3 times higher than maximum serum levels (17), but as this is a local deposit it will not lead to high serum concentrations in patients. We did not test a lower bolus; thus, we do not know if the bolus' success is because of the higher dose. What was most surprising is that we still measured Cxb in the medium at days 14 and 28, although all initial culture medium was removed at day 3. If Cxb was not degraded and distributed evenly throughout the fluid in tissue and medium, only approximately 70% of Cxb was removed at each medium change. Therefore, the bolus is likely to provide an effective dose longer than 3 days, possibly even until day 14, but not until day 28. Cxb only decreased 50% between days 14 and 28, where a 99% decrease was expected, so it is probable that Cxb is binding to the tissue. In blood serum samples, 97% of Cxb is bound to serum albumin (17), which is also a component of our culture medium. Furthermore, albumin is detected in osteoarthritic cartilage tissue, bound to keratan sulfate, one of the main sGAGs in the disc, through disulfide bonds (37). If this proposed mechanism of albumin binding to the tissue is correct, this will have clinical implications. In 80% of extruded herniation samples, blood vessels are observed (38) so, Cxb injected adjacent to extruded NP tissue can diffuse in and bind to pre-bound albumin, lengthening the therapeutic effect. Possibly, this might explain the relative success of epidural steroid injections, as the NP tissue can prolong the effect of a single drug injection. Nevertheless, more research needs to be done to determine the validity of this mechanism. It is also possible that Cxb is binding to other proteins in the NP. This interesting finding shows the value of using our tissue model for preclinical evaluation of therapies for disc herniation, as in cell culture this property of Cxb would be missed. However, a limitation of this model is the absence of the body immune response. Repeating this study in a suitable animal model will truly show if a single injection of Cxb will be as successful as a sustained release biomaterial. Nevertheless, treatment with a COX2 inhibitor like Cxb has clinical potential, as not only the swelling NP tissues in this study, but also infiltrated samples, produce PGE₂.

Another interesting finding is that the gel alone was able to reduce IL-6 production to some extent, especially at day 14. This reduction was also observed at day 14 with the 10 mM loaded gels but also with the empty gels (Fig. S3), indicating that the biomaterial itself affected IL-6 production. As we used Transwell[®] systems (pore size 0.4 mm) in this experiment, there was no direct contact between tissue and biomaterials, but degradation products could have reached the tissue. When the gels degrade, magnesium ions (Mg₂) leach out, which can affect IL-6 production (39). Furthermore, a relatively large amount of biomaterial was used in the gel groups (500 ml per sample in all concentrations). Nevertheless, any direct effect of the gel on IL-6 production is only partial and transient, and any benefits thereof remain to be investigated.

Conclusion

Exposing NP tissue explants to lower tonicity conditions, without the inflammatory system response, increased PGE₂ and IL-6. These cytokines are interesting candidates when treating acute herniation, as they are produced before infiltration of macrophages, and involved in pain and inflammation. Cxb could successfully stop PGE₂, but not IL-6, production for 28 days when supplied by sustained release biomaterials. Interestingly, a bolus was able to achieve the same result, revealing that NP tissue itself can function as a carrier for sustained release of Cxb.

Acknowledgments

This research forms part of the Project P2.01 IDiDAS of the research program of the BioMedical Materials institute, a Dutch public-private partnership. We would like to acknowledge Paul van Midwoud at InGell Pharma for celecoxib release measurements, Irene Arkesteijn at TU/e for the valuable contribution to the artificial annulus model, Renz van Ee and Klaas Timmer at TNO, and Detlef Schumann at DSM for supplying us with the biomaterials.

Disclosure Statement

The academic partners did not receive direct funding from the commercial partners and only non-commercial authors interpreted the data.

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Table 1. Overview of the culture groups.

Group description	Swelling	Treatment duration (Days)	Delivery
(1) Artificial annulus	Contained by artificial annulus	-	-
(2) Free swelling	Free swelling	-	-
(3) Bolus 10 µM	Free swelling	3	Added to media
(4) Sustained release control 1 µM	Free swelling	28	Added to media
(5) Microspheres empty	Free swelling	-	Microspheres
(6) Microspheres 0.1 µM	Free swelling	28	Microspheres
(7) Microspheres 1 µM	Free swelling	28	Microspheres
(8) Microspheres 10 µM	Free swelling	28	Microspheres
(9) Gel empty	Free swelling	-	Injectable gel
(10) Gel 0.1 µM	Free swelling	28	Injectable gel
(11) Gel 1 µM	Free swelling	28	Injectable gel
(12) Gel 10 µM	Free swelling	28	Injectable gel

Figure 1. Tissue NP explant culture system.

Image of nucleus pulposus (NP) explants in artificial annulus culture. Artificial annulus samples (right) were cultured in a deep-well Transwell® plate with 6 ml of culture medium. As the artificial annulus samples might be buoyant, a stainless steel cylinder was added to the culture insert (left) to keep samples submerged (drawn by Anthal Smits).



Figure 2. Schematic image of NP explants in free swelling culture.

Explants were cultured in a deep-well insert plate (bottom). In some groups, celecoxib (Cxb) was added to the medium directly. Both the injectable gel (top left), and the microspheres (top right) were added using a culture insert. LDH, layered double hydroxide; pNIPAAM, poly(N-isopropylacrylamide); RT, room temperature (drawn by Anthal Smits).



Figure 3. Biochemical content of NP explants after 28 days.

Weight change (A), water content (B), sulfated glycosaminoglycan (sGAG) content expressed per dry weight (dw) (C), fixed charge density in mEq/g (D), DNA content expressed per dw (E) and hydroxyproline content expressed per dw (F). Values are means standard deviation, n = 6. * Different from artificial annulus; p < 0.05.



Figure 4. Cytokine release into the medium over time.

Release of prostaglandin E_2 (PGE₂, **A**) and interleukin-6 (IL-6, **B**), in pg/ml, during 3 days, normalized to original sample wet weight (ww). Values are means +/- standard deviation, n = 6. * Different from artificial annulus at same time point, p < 0.05.



Figure 5. Cxb concentration measured in the medium over time.

Concentration in mM at days 3, 14, 21, and 28 for both biomaterials aimed for 1mM release, and at days 14 and 28 for bolus. Values are means + standard deviation, n = 6. * Different from gel at same time point, # different from previous time point of the same biomaterial, \$ different from 0 mM, p < 0.05.



Figure S1. PGE₂ release into the media.

Release at day 28, in pg/ml, during 3 days, normalized to original sample ww. Values are means \pm standard deviation, n = 6.



Figure S2. Cytokine release into the medium over time.

Release of interleukin-1 β (IL-1 β , a) and tumor necrosis factor α (TNF α , b), in pg/ml, during 3 days, normalized to original sample ww. Values are means \pm standard deviation, n = 6.



Figure S3. IL-6 release into the media.

 $Release \ at \ day \ 14, \ in \ pg/ml, \ during \ 3 \ days, \ normalized \ to \ original \ sample \ ww. \ Values \ are \ means \ \pm \ standard \ deviation, \ n=6.$

