Research Article

Use of Biosynthesized Allopregnanolone as Treatment for Infantile Spasms (West Syndrome)

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Allopregnanolone is a neurosteroid recently shown to improve cerebral conditions such as post traumatic stress disorder, concussions, epilepsies, Alzheimer's, Parkinson's, and Infantile Spasms. It is suggested that Allopregnanolone works by activating signaling pathways and gene expressions that allow neural stem cells to regenerate. However, allopregnanolone, despite its numerous benefits, is not commonly produced, as it has a high cost of production. High purity allopregnanolone costs $143 for 5 mg, and a useful dose is around 500-1,000 mg. The current research focuses on providing a cost-efficient method of producing allopregnanolone. Recent studies show that allopregnanolone may be produced through a series of enzymatic reactions. The current research attempts to produce allopregnanolone from cholesterol, through a biosynthesis process in a bacterial system. The biosynthesis of allopregnanolone would greatly reduce the cost of both producing and buying the drug.

Keywords: Biomanufacturing, pharmaceuticals, Biochemical engineering, Recombinant Biology, Allopregnanolone, West Syndrome, Infantile Spasms, Biosynthesis

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Watch a video introduction by the authors at https://youtu.be/pfQQqz8WEpQ

Allopregnanolone is a neurosteroid known to be made in prepubescent females (Reddy 2005). Typically, once a female reaches puberty, production of this drug is halted. Unfortunately, a small percentage of prepubescent females are allopregnanolone deficient and suffer from epilepsies (Reddy 2005). The only possible treatment available is allopregnanolone supplements, which, much like the allopregnanolone synthesized in the body, activates signaling pathways and gene expressions that allow neural stem cell regeneration (Irwin et al. 2014). Unfortunately, pharmaceutical companies are reluctant to produce allopregnanolone due to a high production cost and small affected population. Currently, allopregnanolone costs approximately $143 per 5 mg, and as a normal dose of this drug is about 500-1,000 mg, the cost would be around $14,300 for one normal dose. As such, the drug was placed under the Angel Act as an orphan drug.

Fortunately, for these individuals, allopregnanolone was recently discovered to be a promising promoter of brain cell regeneration (Irwin et al. 2014). Once applied to test subjects with cerebral conditions, allopregnanolone was found to be crucial in the improvement of conditions such as epilepsies, West Syndrome, post traumatic stress disorder (PTSD), concussions, Alzheimer's, multiple sclerosis, Niemann-Pick, diabetic neuropathy, and Parkinson's, among others (Irwin et al. 2014). An experiment carried out on mice with Alzheimer's found that allopregnanolone reduced β-amyloid and neuroinflammatory symptoms (Irwin et al. 2014). In addition, allopregnanolone was also discovered to activate signaling pathways and gene expression for the regeneration of neural stem cells (Irwin et al. 2014). Research has also shown that allopregnanolone may be a useful tool in treating traumatic brain injuries, as there is experimental evidence that allopregnanolone reduces the inflammatory cytokines produced by such injuries (He et al. 2004).

Interestingly, allopregnanolone was also discovered to be reduced in those individuals associated with depression or other psychiatric disorders (Bäckström et al. 2014; Schüle et al.

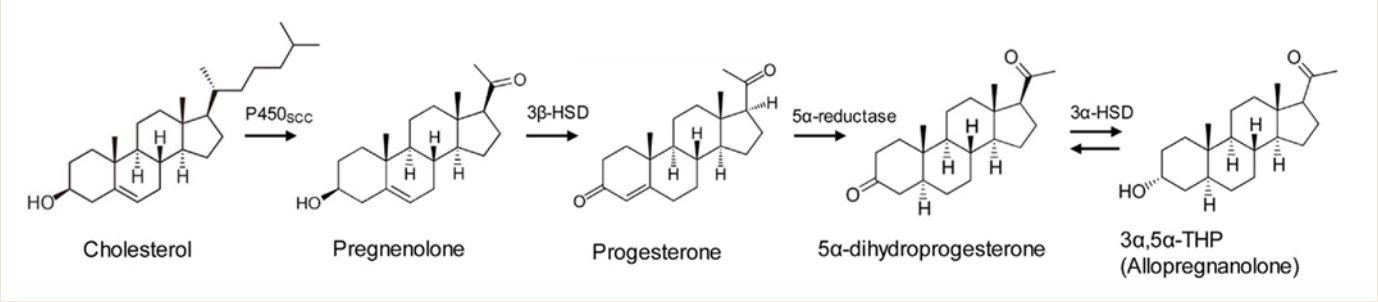

Figure 1. *Biosynthesis of allopregnanolone.*

2014). Specifically, reduced allopregnanolone levels in peripheral blood or cerebrospinal fluid correlated with depression, anxiety, premenstrual dysphoric disorder, schizophrenia, and impulsive aggression (Schüle et al. 2014). When antidepressants were introduced, allopregnanolone levels were also found to increase. This may suggest that the presence of allopregnanolone may be responsible for an individual's depression; however, this has not been confirmed. In an experiment, in which mice were socially isolated from birth for approximately six weeks, allopregnanolone was seen to decrease by 50% (Dong et al. 2001). This may correlate and even validate the idea that a decrease in allopregnanolone is associated with depression.

Due to the newly discovered uses for Allopregnanolone, multiple individuals and organizations have attempted to facilitate the production of the neurosteroid; thus assisting both the allopregnanolone-deficient prepubescent females and the thousands of individuals suffering from neurological diseases. Currently, the formulations of allopregnanolone for a diverse array of administration routes have been created for preclinical and clinical testing. The successful development and implementation of a relatively cheap and easy method of allopregnanolone production would allow for better and larger scale production of this now "miracle drug."

Naturally, the brain and peripheral nerves are capable of the biosynthesis of allopregnanolone. Initially, the peripheral gland produces progesterone, and through the introduction of 5α-reductase and 3α-hydroxysteroid dehydrogenase, it may be converted into allopregnanolone (Frye et al. 2014). In addition, allopregnanolone can also be biosynthesized in the brain alone. Essentially, in this process, translocator and steroidogenic acute regulatory proteins transport cholesterol into mitochondria (Frye et al. 2014). Then, it is oxidized by cytochrome P450-dependent C27 side chain cleavage enzymes into pregnenolone (Frye et al. 2014). Pregnenolone is then converted to progesterone by 3β-HSD enzymes, and finally converted to allopregnanolone by the 5α-reductase and 3α-HSD (Frye et al. 2014).

We believe that allopregnanolone can be cheaply produced by reconstructing the naturally occurring biosynthesis procedure for allopregnanolone in a bacterial system. Basically, our system will replicate the four-gene biosynthesis pathway shown in Figure 1. First, bacteria producing the p450scc enzyme will convert cholesterol to pregnenolone. Then, pregnenolone will be transformed into progesterone by bacteria expressing 3β-HSD. Likewise, 5α-dihydroprogesterone will be produced from progesterone by bacteria that make the 5α-reductase enzyme. Finally, 5α-dihydroprogesterone will be transformed into allopregnanolone by bacteria possessing a gene for the 5α-THP enzyme.

The step-by-step biosynthesis of allopregnanolone can be coordinated by encapsulating bacteria containing the required biosynthesis genes in polyacrylamide beads and layering the beads in the required order. Using a purification column, as shown in Figure 2, products can be extracted using a step-by-step synthesis. In the proposed system, there will be fewer beads in the final step, which involves the 3α-HSD catalyzed conversion of progesterone to allopregnanolone. This is intentionally planned so the mass build up of allopregnanolone will not drive the reaction backwards.

In preparation for constructing our allopregnanolone biosynthesis column, we used yeast to evaluate the effects of polyacrylamide encapsulation on microbial metabolism.

Materials and Methods

Laboratory Safety. This project will only require a biosafety level of one. Sterile techniques will be used in order to guarantee the success of this experiment. Alcohol, gloves, bleach, and an autoclave will also be used to maintain sanitary conditions.

Production of Polyacrylamide Calcium Alginate Microbeads. We began by adding 1 g sodium alginate to a bowl containing 236 mL water. We used a hand mixer to combine the two ingredients thoroughly. Once the sodium alginate was fully dissolved, we let the solution sit for 15 min. to ensure there were no air bubbles. Next, we added 0.5 g yeast for every 9.5 mL of sodium alginate. We dissolved calcium lactate in a separate bowl and added enough polyacrylamide to make a 4% solution. 5 g calcium lactate were added to the large bowl holding 946 mL water. We mixed the solution well using an ordinary whisk or mixing spoon to ensure the calcium lactate was completely dissolved. We used a transfer pipette to gently transfer the sodium alginate-yeast solution, dropwise, into the calcium lactate bath. The bath immediately started to form spheres. Once the spheres had taken shape, the solution was gently stirred for about 3 min. To aid the forming of the gel, the beads were then rinsed with 10 mL sterile water in an incubator set to 37°C, containing 10 µL tetramethylethylenediamine (TEMED), in order to catalyze the polymerization of acrylamide, and 50 µL of ammonium persulfate. The beads were rinsed with sterile water over a slotted spoon. This produced beads that were around 5 mm in diameter.

Diffusion of Food Coloring in Polyacrylamide Beads. 12 beads were placed in a Falcon tube with 10 mL water and two

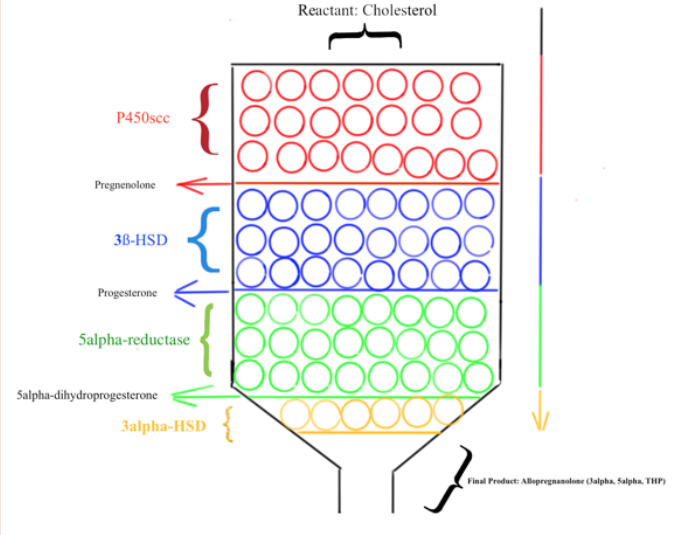

Figure 2. *Theoretical synthesis of allopregnanolone in a purification column with layered polyacrylamide beads with sequential enzymes.*

drops of green food coloring. The Falcon tubes were placed in a rotator, and three beads were taken after 5, 10, and 15 min. The beads were placed in a microcentrifuge tube and spun down for 5 min. at 10,000 RPM. Using a 1 mL pipette, the supernatant was removed and tested in a spectrometer at an absorbance of 500 nm.

Serial Dilution and Benedict's Solution Test. A 1:10 serial dilution was performed on a 20% dextrose solution. Serial dilutions were performed in total by taking 1 mL of the first tube and adding it to a second tube that contained 9 mL of water. We repeated the process for the remaining four tubes. After the dilution, the different tubes were placed in boiling water for 10 min. for the reaction to occur.

Fermentation Test. A 2% dextrose solution was used for the fermentation reactions. 0.5 g of free yeast and 0.5 g of yeast captured in the microbeads were placed in 9.5 mL of 2% dextrose solution respectively. We used four tubes for each treatment. Each culture (free yeast cells and yeast cells captured in microbeads) were placed in a rotatory incubator at 30°C. Each treatment had four tubes. One culture from each was removed after 5, 10, 15, and 20 min. of incubation. The removed cultures were tested by Benedict's reaction after the microbead and the free cells were removed from each tube. The free yeast was removed by centrifugation at 6,000 RPM for 5 min. in the cold, and the beads were removed by filtration and kept in the fridge until testing.

Table 1. **Diffusion represented by absorption of acrylamide and normal beads.**

Trial	Absorption of Acrylamide Beads (AU)			Absorption of Normal Beads (AU)		
	20 min.	40 min.	60 min.	20 min.	40 min.	60 min.
1	0.25	0.28	0.33	0.20	0.23	0.33
2	0.28	0.31	0.35	0.23	0.26	0.35
3	0.31	0.32	0.34	0.21	0.25	0.34

Results

Measuring the Abilities of Microbeads to Absorb Small Molecules. Absorption gives insight to any negative effects the polyacrylamide would have on the diffusion of small molecules through the beads. Yielded data suggests that there were no differences compared to the control (microbeads with no polyacrylamide).

Figure 3 shows the absorption of food coloring into a microbead. Table 1 reports the data obtained using a spectrometer at 500 nm. Adding polyacrylamide increased the strength of the beads dramatically. The beads allowed for easy diffusion of small molecules such as dextrose and food coloring but not large molecules such as starch (data not shown).

Evaluating the Limits of Detection for Benedict's Solution. Benedict's solution sugar test can provide a demonstration of not only the presence of sugar, but also the metabolism of a microorganism, and the optimal concentration to asses this metabolism of dextrose. A serial dilution test was employed to see the limits of detection for Benedict's solution. Figure 4 shows the serial dilution of of 20% dextrose after Benedict's solution test. Blue represents very little or no dextrose present.

Figure 3. *Diffusion of food coloring in acrylamide microbeads.*

Comparing the Metabolic Rates of Free-Floating and Microencapsulated Yeast. In the fermentation test, we wanted to compare the metabolism of dextrose by yeast that is free floating and yeast that has been encapsulated. We decided to use yeast instead of bacteria because it was readily available from the store, easy to measure, and can convert sugar to alcohol as a byproduct. We used Benedict's solution to observe the disappearance of dextrose at different time intervals.

Figure 5A shows the free-floating and microencapsulated yeast that were used in this experiment. Figure 5B shows the Benedict's reaction of free cell fermentation. In this experiment, the cells were centrifuged and the supernatant was transferred to a new Falcon tube for the reaction. Figure 3C shows the Benedict's reaction for microencapsulated yeast. The beads were removed from solution before the reaction took place.

We also wanted to test the production of alcohol, but it seems we need to have a license to do that. We had tried to order a

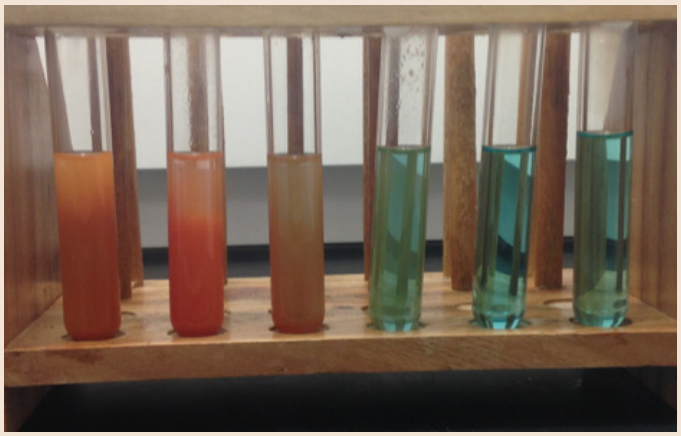

Figure 4. *Presence of dextrose gradient indicated by Benedict's solution.*

probe, but our school administrator told us that we need to get a site alcohol license for the research.

Discussions

This methodology test gives insight on specific protocols and procedures to be used in the synthesis of allopregnanolone. As opposed to an industrial bioreactor, biobeads can provide compartmentalization, and most importantly, a faster synthesis. This is displayed by the fact that in industrial bioreactors, cells must grow before they are capable to synthesize. Using fermentation as a model, the rate of fermentation was significantly faster using polyacrylamide beads as opposed to free yeast cells (20 min. vs. 5-10 min.). Benedict's test turns the solution blue when no sugars are present. Once again, the free yeast cells had to grow before fully converting dextrose into ethanol and can be accounted for in the lag, whereas the polyacrylamide beads were able to immediately ferment, and therefore completely fermented at a much faster rate. In regards to the synthesis of allopregnanolone, the use of biobeads allow for a mass accumulation of product which, in a biosynthesis perspective, drives the reversible synthesis of allopregnanolone from 5α-dihidroprogestorone forward.

An additional benefit of the polyacrylamide beads is increased strength, as measured using an instrument which places all force on the bead itself. Upon measurement, a single polyacrylamide bead was able to withstand more than 50.00 g of exerted force, over 2.5 times more than the regular counterpart.

Feasibility of the synthesis was also indicated by assessing the rates of diffusion of biobeads versus their regular bead counterpart. Diffusion was assessed by evaluating relative absorption food coloring. Regular and polyacrylamide beads had virtually the same absorption (0.25 to 0.32 AU). This implies that small molecules are able to diffuse freely through the beads and large, chained molecules are not able to. Thus, in a series of reactions, cholesterol will likely be able to diffuse through the beads and react with appropriate enzymes. The benefit of the biobeads is not only demonstrated through a faster synthesis ability and feasible diffusion, but also due to the durability of the bead; more beads can be stacked without the threat of collapse.

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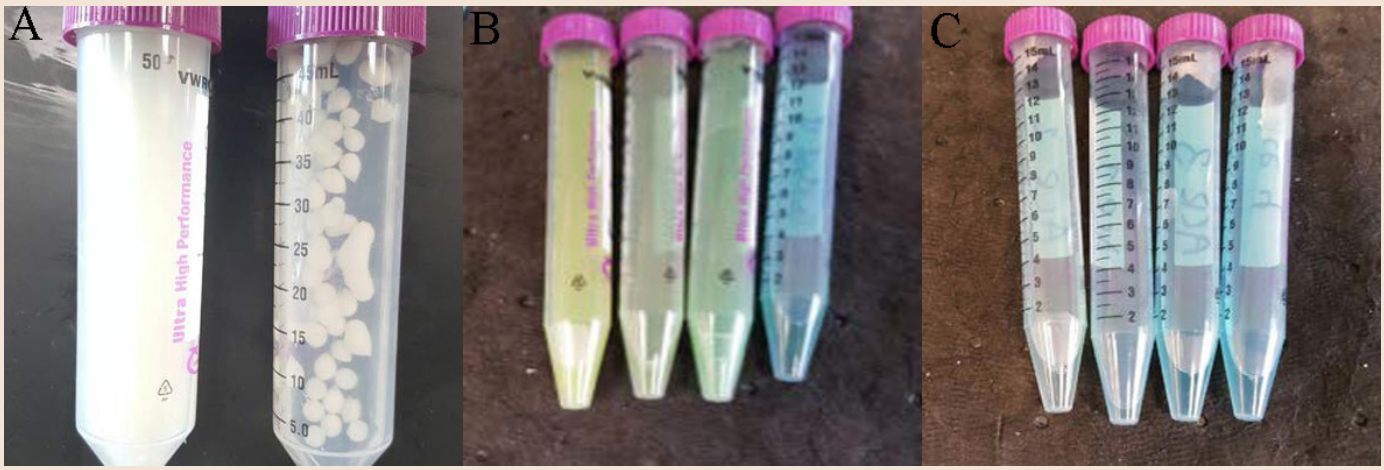

Figure 5. *Free yeast and yeast encapsulated in micro-bio-beads were used in this experiment (A). The rates of fermentation for free yeast cells (B) and yeast encapsulated in microbeads (C) are shown from left to right in Benedict's solution (5, 10, 15, 20 min.).*

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